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(54) Title: METHOD AND PRODUCT FOR REGULATING CELL RESPONSIVENESS TO EXTERNAL SIGNALS			
(57) Abstract The present invention relates to isolated MEKK proteins, nucleic acid molecules having sequences that encode such proteins, and antibodies raised against such proteins. The present invention also includes methods to use such proteins to regulate signal transduction in a cell. The present invention also includes therapeutic compositions comprising such proteins or nucleic acid molecules that encode such proteins and their use to treat animals having medical disorders including cancer, inflammation, neurological disorders, autoimmune diseases, allergic reactions, and hormone-related diseases. When MEKK is expressed, it phosphorylates and activates MEKs including MEK-1, MEK-2 and JEK.			

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METHOD AND PRODUCT FOR REGULATING CELL
RESPONSIVENESS TO EXTERNAL SIGNALS

FIELD OF THE INVENTION

This invention relates to isolated nucleic acid
5 molecules encoding MEKK proteins, substantially pure MEKK
proteins, and products and methods for regulating signal
transduction in a cell.

SUMMARY OF THE INVENTION

The present invention relates to a substantially pure
10 MEKK protein capable of phosphorylating mammalian MEK
protein, in which the MEKK protein comprises a catalytic
domain. The present invention includes a substantially
pure MEKK protein capable of regulating signals initiated
from a growth factor receptor on the surface of a cell by
15 regulating the activity of MAPK protein, the ability to
regulate being divergent from Raf protein signal
regulation. In particular, the substantially pure MEKK
protein comprises at least a portion of an amino acid
sequence encoded by a nucleic acid sequence that is capable
20 of hybridizing under stringent conditions with a nucleic
acid molecule encoding an amino acid sequence including SEQ
ID NO:4, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:10. The
substantially pure MEKK protein capable of regulating the
activity of MAPK protein, said protein having an amino acid
25 sequence distinct from Raf protein.

The present invention also includes a formulation
comprising at least one isolated protein having at least a
portion of an amino acid sequence encoded by a nucleic acid
sequence that is capable of hybridizing under stringent
30 conditions with a nucleic acid molecule encoding an amino

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acid sequence including SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:10.

One aspect of the present invention includes an isolated nucleic acid molecule having a sequence encoding
5 a protein capable of phosphorylating mammalian MEK independent of Raf protein and capable of regulating the activity of MAPK protein. In particular, the present invention includes an isolated nucleic acid molecule capable of hybridizing under stringent conditions with a
10 nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and SEQ ID NO:9.

Another aspect of the present invention includes a recombinant molecule, comprising a nucleic acid molecule
15 capable of hybridizing under stringent conditions with a nucleic acid sequence including SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and SEQ ID NO:9, in which the nucleic acid molecule is operatively linked to an expression vector.

20 Yet another aspect of the present invention is a recombinant cell transformed with a recombinant molecule, comprising a nucleic acid molecule operatively linked to an expression vector, the nucleic acid molecule comprising a nucleic acid sequence capable of hybridizing under
25 stringent conditions with a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and SEQ ID NO:9 (i.e., the nucleic

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acid sequence shown in Table 1, Table 2, Table 3, Table 4 and Table 5).

The present invention also includes a method for regulating the homeostasis of a cell comprising regulating the activity of an MEKK-dependent pathway relative to the activity of a Raf-dependent pathway in the cell. In particular, the method comprises regulating the apoptosis of the cell. Such a method is useful for the treatment of a medical disorder. In particular, the method is useful for inhibiting tumorigenesis and autoimmunity.

According to the present invention, the method for treatment of a disease, comprises administering to a patient an effective amount of a therapeutic compound comprising at least one regulatory molecule including a molecule capable of decreasing the activity of a Raf-dependent pathway, a molecule capable of increasing the activity of an MEKK-dependent pathway, and combinations thereof, in which the effective amount comprises an amount which results in the depletion of harmful cells involved in the disease.

Also included in the present invention is a therapeutic compound capable of regulating the activity of an MEKK-dependent pathway in a cell identified by a process, comprising: (a) contacting a cell with a putative regulatory molecule; and (b) determining the ability of the putative regulatory compound to regulate the activity of an MEKK-dependent pathway in the cell by measuring the

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activation of at least one member of said MEKK-dependent pathway.

One embodiment of the present invention includes a substantially pure protein, in which the protein is isolated using an antibody capable of selectively binding to an MEKK protein capable of phosphorylating mammalian MEK protein and capable of regulating the activity of MAPK protein independent of Raf protein, the antibody capable of being produced by a method comprising: (a) administering to an animal an effective amount of a substantially pure MEKK protein of the present invention; and (b) recovering an antibody capable of selectively binding to the MEKK protein.

Another embodiment of the present invention includes an isolated antibody capable of selectively binding to an MEKK protein, the antibody capable of being produced by a method comprising administering to an animal an effective amount of a substantially pure protein of the present invention, and recovering an antibody capable of selectively binding to the MEKK protein.

BACKGROUND OF THE INVENTION

Mitogen-activated protein kinase (MAPKs) (also called extracellular signal-regulated kinases or ERKs) are rapidly activated in response to ligand binding by both growth factor receptors that are tyrosine kinases (such as the epidermal growth factor (EGF) receptor) and receptors that are coupled to heterotrimeric guanine nucleotide binding proteins (G proteins) such as the thrombin receptor. The

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MAPKs appear to integrate multiple intracellular signals transmitted by various second messengers. MAPKs phosphorylate and regulate the activity of enzymes and transcription factors including the EGF receptor, Rsk 90, phospholipase A₂, c-Myc, c-Jun and Elk-1/TCF. Although the rapid activation of MAPKs by receptors that are tyrosine kinases is dependent on Ras, G protein-mediated activation of MAPK appears to occur through pathways dependent and independent of Ras.

Complementation analysis of the pheromone-induced signaling pathway in yeast has defined a protein kinase system that controls the activity of Spk1 and Fus3-Kss1, the *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* homologs of MAPK (see for example, B.R. Cairns et al., *Genes and Dev.* 6, 1305 (1992); B.J. Stevenson et al., *Genes and Dev.* 6, 1293 (1992); S.A. Nadin-Davis et al., *EMBO J.* 7, 985 (1988); Y. Wang et al., *Mol. Cell. Biol.* 11, 3554 (1991). In *S. cerevisiae*, the protein kinase Ste7 is the upstream regulator of Fus3-Kss1 activity; the protein kinase Ste11 regulates Ste7. The *S. pombe* gene products Byr1 and Byr2 are homologous to Ste7 and Ste11, respectively. The MEK (MAPK Kinase or ERK Kinase) or MKK (MAP Kinase kinase) enzymes are similar in sequence to Ste7 and Byr1. The MEKs phosphorylate MAPKs on both tyrosine and threonine residues which results in activation of MAPK. The mammalian serine-threonine protein kinase Raf phosphorylates and activates MEK, which leads to activation of MAPK. Raf is activated in response to growth factor

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receptor tyrosine kinase activity and therefore Raf may activate MAPK in response to stimulation of membrane-associated tyrosine kinases. Raf is unrelated in sequence to *Stell* and *Byr2*. Thus, Raf may represent a divergence in mammalian cells from the pheromone-responsive protein kinase system defined in yeast. Cell and receptor specific differences in the regulation of MAPKs suggest that other Raf independent regulators of mammalian MEKs exist.

Certain biological functions, such as growth and differentiation, are tightly regulated by signal transduction pathways within cells. Signal transduction pathways maintain the balanced steady state functioning of a cell. Disease states can arise when signal transduction in a cell breaks down, thereby removing the tight control that typically exists over cellular functions. For example, tumors develop when regulation of cell growth is disrupted enabling a clone of cells to expand indefinitely. Because signal transduction networks regulate a multitude of cellular functions depending upon the cell type, a wide variety of diseases can result from abnormalities in such networks. Devastating diseases such as cancer, autoimmune diseases, allergic reactions, inflammation, neurological disorders and hormone-related diseases can result from abnormal signal transduction.

Despite a long-felt need to understand and discover methods for regulating cells involved in various disease states, the complexity of signal transduction pathways has precluded the development of products and processes for

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regulating cellular function by manipulating signal transduction pathways in a cell. As such, there remains a need for products and processes that permit the implementation of predictable controls of signal
5 transduction in cells, thus enabling the treatment of various diseases that are caused by abnormal cellular function.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a schematic representation of the signal
10 pathways of vertebrates and yeast.

Fig. 2 is a schematic representation of the dual MEKK and Raf pathways divergent from Ras protein pathway.

Fig. 3A shows a Northern (RNA) blot of a single 7.8 kb MEKK mRNA in several cell lines and mouse tissues.

15 Fig. 3B shows a Southern (DNA) blot of the MEKK gene.

Fig. 3C shows an immunoblot showing expression of the 78 kD and 50 kD forms of MEKK in rodent cell lines.

Fig. 4 shows immunoprecipitates of MEKK protein using MEKK antiserum.

20 Fig. 5 shows immunoblotting of MEKK protein in immunoprecipitates and cell lysates.

Fig. 6A shows the activation of MAPK in COS cells transfected with MEKK.

Fig. 6B is an immunoblot showing expression of MEKK in
25 cells either treated or not treated with EGF.

Fig. 7 shows the activation and phosphorylation of MEK in COS cells transfected with MEKK.

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Fig. 8A shows the phosphorylation of MEK-1 by MEKK.

Fig. 8B shows the time course of phosphorylation of MEK-1 by MEKK expressed in COS cells.

Fig. 8C is an immunoblot of MEKK overexpressed in COS
5 cells.

Fig. 9A shows the phosphorylation of MAPK by activated MEK-1.

Fig. 9B shows phosphorylation of MEK-1 by immunoprecipitated MEKK.

10 Fig. 10A shows the phosphorylation of MEK-1 by activated Raf.

Fig. 10B shows the phosphorylation state of Raf isolated from COS cells which are overexpressing MEKK and have been treated with EGF.

15 Fig. 11 shows the relative ability of immunoprecipitated MEKK and Raf-B to phosphorylate kinase inactive MEK-1.

Fig. 12 shows a time course of EGF-stimulated MEKK and Raf-B activation.

20 Fig. 13 shows that the immunodepletion of Raf-B from MEKK immunoprecipitates has no effect on MEKK activity.

Fig. 14 shows that the immunodepletion of Raf-B from MEKK immunoprecipitates decreases Raf-B activity.

25 Fig. 15 shows MEKK activity in FPLC Mono Q ion-exchange column fractions of PC12 cell lysates.

Fig. 16 shows inhibition of MEKK and Raf-B activation by dominant negative N¹⁷RAS expression.

Fig. 17 shows activation of MEK protein by 98 kD MEKK.

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Fig. 18 shows inhibition of EGF activation of MEKK by forskolin.

Fig. 19 shows improved MEKK activity by truncated MEKK molecules.

5 Fig. 20 shows JNK activation by MEKK protein.

Fig. 21 shows regulation of c-Myc controlled transcription and not CREB controlled transcription by MEKK protein.

10 Fig. 22 is a schematic representation of MEKK regulation of c-Myc controlled transcription.

Fig. 23 shows induction of p38 MAPK phosphorylation by MEKK 3.

Fig. 24 shows induction of cellular apoptosis in Swiss 3T3 and REF52 cells by beauvericin.

15 Fig. 25 shows induction of cellular apoptosis in REF52 cells by MEKK.

Fig. 26 shows induction of cellular apoptosis in Swiss 3T3 and REF52 cells by MEKK.

20 Fig. 27 shows 3 representative microscopic views of apoptotic REF52 cells expressing MEKK protein.

Fig. 28 shows 3 representative microscopic views of apoptotic Swiss 3T3 cells expressing MEKK protein.

Fig. 29 shows similar stimulation of MAPK activity by MEKK protein and Raf protein.

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DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a novel mitogen ERK kinase kinase protein (MEKK) capable of regulating signal

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transduction in cells. The present invention includes a novel method for treating disease by regulating the activity of cells involved in such disease. The present invention is particularly advantageous in that the novel product and method of the present invention is capable of regulating a signal transduction pathway that can lead to cellular apoptosis.

One embodiment of the present invention is an isolated MEKK protein. According to the present invention, an isolated protein is a protein that has been removed from its natural milieu. An isolated MEKK protein can, for example, be obtained from its natural source, be produced using recombinant DNA technology, or be synthesized chemically. As used herein, an isolated MEKK protein can be a full-length MEKK protein or any homologue of such a protein, such as an MEKK protein in which amino acids have been deleted (e.g., a truncated version of the protein, such as a peptide), inserted, inverted, substituted and/or derivatized (e.g., by glycosylation, phosphorylation, acetylation, myristoylation, prenylation, palmitoylation, amidation and/or addition of glycosylphosphatidyl inositol), wherein the modified protein is capable of phosphorylating mitogen ERK kinase (MEK) and/or Jun ERK kinase (JEK). A homologue of an MEKK protein is a protein having an amino acid sequence that is sufficiently similar to a natural MEKK protein amino acid sequence that a nucleic acid sequence encoding the homologue is capable of hybridizing under stringent conditions to (i.e., with) a

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nucleic acid sequence encoding the natural MEKK protein amino acid sequence. As used herein, stringent hybridization conditions refer to standard hybridization conditions under which nucleic acid molecules, including oligonucleotides, are used to identify similar nucleic acid molecules. Such standard conditions are disclosed, for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press, 1989. A homologue of an MEKK protein also includes a protein having an amino acid sequence that is sufficiently cross-reactive such that the homologue has the ability to elicit an immune response against at least one epitope of a naturally-occurring MEKK protein.

The minimal size of a protein homologue of the present invention is a size sufficient to be encoded by a nucleic acid molecule capable of forming a stable hybrid with the complementary sequence of a nucleic acid molecule encoding the corresponding natural protein. As such, the size of the nucleic acid molecule encoding such a protein homologue is dependent on nucleic acid composition, percent homology between the nucleic acid molecule and complementary sequence, as well as upon hybridization conditions per se (e.g., temperature, salt concentration, and formamide concentration). The minimal size of such nucleic acid molecules is typically at least about 12 to about 15 nucleotides in length if the nucleic acid molecules are GC-rich and at least about 15 to about 17 bases in length if they are AT-rich. As such, the minimal size of a nucleic

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acid molecule used to encode an MEKK protein homologue of the present invention is from about 12 to about 18 nucleotides in length. There is no limit, other than a practical limit, on the maximal size of such a nucleic acid molecule in that the nucleic acid molecule can include a portion of a gene, an entire gene, or multiple genes, or portions thereof. Similarly, the minimal size of an MEKK protein homologue of the present invention is from about 4 to about 6 amino acids in length, with preferred sizes depending on whether a full-length, multivalent protein (i.e., fusion protein having more than one domain each of which has a function), or a functional portion of such a protein is desired.

MEKK protein homologues can be the result of allelic variation of a natural gene encoding an MEKK protein. A natural gene refers to the form of the gene found most often in nature. MEKK protein homologues can be produced using techniques known in the art including, but not limited to, direct modifications to a gene encoding a protein using, for example, classic or recombinant DNA techniques to effect random or targeted mutagenesis. The ability of an MEKK protein homologue to phosphorylate MEK and/or JEK protein can be tested using techniques known to those skilled in the art. Such techniques include phosphorylation assays described in detail in the Examples section.

In one embodiment, an MEKK protein of the present invention is capable of regulating an MEKK-dependent

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pathway. According to the present invention, an MEKK-dependent pathway refers generally to a pathway in which MEKK protein regulates a pathway substantially independent of Raf, and a pathway in which MEKK protein regulation converges with common members of a pathway involving Raf protein, in particular, MEK protein (see Fig. 2). A suitable MEKK-dependent pathway includes a pathway involving MEKK protein and JEK protein, but not Raf protein. One of skill in the art can determine that regulation of a pathway by an MEKK protein is substantially independent of Raf protein by comparing the ability of an MEKK protein and a Raf protein to regulate the phosphorylation of a downstream member of such pathway using, for example, the general method described in Example 16. An MEKK protein regulates a pathway substantially independently of Raf protein if the MEKK protein induces phosphorylation of a member of the pathway downstream of MEKK (e.g., proteins including JEK, JNK, Jun and/or ATF-2) by an amount significantly greater than that seen when Raf protein is utilized. For example, MEKK induction of phosphorylation of JNK is preferably at least about 10-fold, more preferably at least about 20-fold and even more preferably at least about 30-fold, greater phosphorylation of JNK protein than the phosphorylation induced when using Raf protein. If MEKK induction of phosphorylation is similar to Raf protein induction of phosphorylation, then one of skill in the art can conclude that regulation of a pathway by an MEKK protein includes members of a signal

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transduction pathway that could also include Raf protein. For example, MEKK induction of phosphorylation of MAPK is of a similar magnitude as induction of phosphorylation with Raf protein.

5 A "Raf-dependent pathway" can refer to a signal transduction pathway in which Raf protein regulates a signal transduction pathway substantially independently of MEKK protein, and a pathway in which Raf protein regulation converges with common members of a pathway involving MEKK
10 protein. The independence of regulation of a pathway by a Raf protein from regulation of a pathway by an MEKK protein can be determined using methods similar to those used to determine MEKK independence.

 In another embodiment, an MEKK protein is capable of
15 regulating the activity of signal transduction proteins including, but not limited to, mitogen ERK kinase (MEK), mitogen activated protein kinase (MAPK), transcription control factor (TCF), Ets-like-1 transcription factor (Elk-1), Jun ERK kinase (JEK), Jun kinase (JNK), stress
20 activated MAPK proteins, Jun, activating transcription factor-2 (ATF-2) and/or Myc protein. As used herein, the "activity" of a protein can be directly correlated with the phosphorylation state of the protein and/or the ability of the protein to perform a particular function (e.g.,
25 phosphorylate another protein or regulate transcription). Preferred MEK proteins regulated by an MEKK protein of the present invention include MEK-1 and/or MEK-2. Preferred MAPK proteins regulated by an MEKK protein of the present

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invention include p38 MAPK, p42 MAPK and/or p44 MAPK. A preferred MEKK protein that is capable of phosphorylating p38 MAPK protein includes a protein encoded by the nucleic acid sequence represented by SEQ ID NO:5 with a protein
5 having the amino acid sequence represented by SEQ ID NO:7 being more preferred. Preferred stress activated MAPK proteins regulated by an MEKK protein of the present invention include Jun kinase (JNK), stress activated MAPK- α and/or stress activated MAPK- β . An MEKK protein of the
10 present invention is capable of increasing the activity of an MEK protein over basal levels of MEK (i.e., levels found in nature when not stimulated). For example, an MEKK protein is preferably capable of increasing the phosphorylation of an MEK protein by at least about 2-fold,
15 more preferably at least about 3-fold, and even more preferably at least about 4-fold over basal levels when measured under conditions described in Example 9.

A preferred MEKK protein of the present invention is also capable of increasing the activity of an MAPK protein
20 over basal levels of MAPK (i.e., levels found in nature when not stimulated). For example, an MEKK protein of the present invention is preferably capable of increasing MAPK activity at least about 2-fold, more preferably at least about 3-fold, and even more preferably at least about 4-
25 fold over basal activity when measured under the conditions described in Example 3.

Moreover, an MEKK protein of the present invention is capable of increasing the activity of a JNK protein. JNK

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regulates the activity of the transcription factor JUN which is involved in controlling the growth and differentiation of different cell types, such as T cells, neural cells or fibroblasts. JNK shows structural and regulatory homologies with MAPK. For example, an MEKK protein of the present invention is preferably capable of inducing the phosphorylation of JNK protein at least about 30 times more than Raf, more preferably at least about 40 times more than Raf, and even more preferably at least about 50 times more than Raf, when measured under conditions described in Example 16.

A preferred MEKK protein of the present invention is additionally capable of inducing the phosphorylation of a c-Myc transcriptional transactivation domain protein in such a manner that the phosphorylated transcriptional transactivation domain of c-Myc is capable of regulating gene transcription. The ability of an MEKK protein to regulate phosphorylation of a c-Myc transcriptional transactivation domain protein exceeds the ability of Raf protein or cyclic AMP-dependent protein kinase to regulate a c-Myc protein. For example, an MEKK protein of the present invention is preferably capable of inducing luciferase gene transcription by phosphorylated c-Myc transcriptional transactivation domain protein at least about 25-fold, more preferably at least about 35-fold, and even more preferably at least about 45-fold, over Raf induction when measured under the conditions described in Example 17.

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Another aspect of the present invention relates to the ability of MEKK activity to be stimulated by growth factors including, but not limited to, epidermal growth factor (EGF), neuronal growth factor (NGF), tumor necrosis factor (TNF), C5A, interleukin-8 (IL-8), monocyte chemotactic protein 1 (MIP1 α), monocyte chemoattractant protein 1 (MCP-1), platelet activating factor (PAF), N-Formyl-methionyl-leucyl-phenylalanine (FMLP), leukotriene B₄ (LTB₄R), gastrin releasing peptide (GRP), IgE, major histocompatibility protein (MHC), peptide, superantigen, antigen, vasopressin, thrombin, bradykinin and acetylcholine. In addition, the activity of an MEKK protein of the present invention is capable of being stimulated by compounds including phorbol esters such as TPA. A preferred MEKK protein is also capable of being stimulated by EGF, NGF and TNF (especially TNF α).

Preferably, the activity of an MEKK protein of the present invention is capable of being stimulated at least 2-fold over basal levels (i.e., levels found in nature when not stimulated), more preferably at least about 4-fold over basal levels and even more preferably at least about 6-fold over basal levels, when a cell producing the MEKK protein is contacted with EGF under the conditions described in Example 3.

Similarly, the activity of an MEKK protein of the present invention is capable of being stimulated at least 1-fold over basal levels, more preferably at least about 2-fold over basal levels and even more preferably at least

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about 3-fold over basal levels by NGF stimulation, when a cell producing the MEKK protein is contacted with NGF under the conditions described in Example 9.

Preferably, an MEKK protein of the present invention
5 is capable of being stimulated at least 0.5-fold over basal levels, more preferably at least about 1-fold over basal levels and even more preferably at least about 2-fold over basal levels by TPA stimulation when a cell producing the MEKK protein is contacted with TPA under the conditions
10 described in Example 9.

TNF is capable of regulating cell death and other functions in different cell types. The present inventor discovered that MEKK stimulation by TNF is independent of Raf. Similarly, the present inventor is the first to
15 appreciate that an MEKK protein can be directly stimulated by ultraviolet light (UV) damage of cells while a Raf-dependent pathway cannot. Therefore, both TNF and UV stimulate MEKK activity without substantially activating Raf. In addition, both UV and TNF activation of MEKK is
20 Ras dependent.

Another aspect of the present invention is the recognition that an MEKK protein of the present invention is capable of regulating the apoptosis of a cell, an ability not shared by Raf protein. As used herein,
25 apoptosis refers to the form of cell death that comprises: progressive contraction of cell volume with the preservation of the integrity of cytoplasmic organelles; condensation of chromatin, as viewed by light or electron

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microscopy; and DNA cleavage, as determined by centrifuged sedimentation assays. Cell death occurs when the membrane integrity of the cell is lost and cell lysis occurs. Apoptosis differs from necrosis in which cells swell and eventually rupture.

A preferred MEKK protein of the present invention is capable of inducing the apoptosis of cells, such that the cells have characteristics substantially similar to cytoplasmic shrinkage and/or nuclear condensation as shown in Figs. 24, 25, 26, 27 and 28. The apoptotic cells in Figs. 24 through 28 were obtained when cells were microinjected with expression plasmids encoding MEKK protein. Injected cells were identified using anti- β -Gal antibody and the DNA of the cells were stained with propidium iodide. Cytoplasmic organization was monitored using an anti-tubulin antibody. The cells were then imaged by differential fluorescent imaging microscopy using techniques standard in the art. The cells demonstrated apoptosis by displaying a morphology having cytoplasmic shrinkage and nuclear condensation.

A schematic representation of the cell growth regulatory signal transduction pathway that is MEKK dependent is shown in Fig. 2. An MEKK protein of the present invention is capable of regulating the activity of JEK protein, JNK protein, Jun protein and/or ATF-2 protein, and Myc protein, such regulation being substantially, if not entirely, independent of Raf protein. Such Raf-independent regulation can regulate the growth

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characteristics of a cell, including the apoptosis of a cell. In addition, an MEKK protein of the present invention is capable of regulating the activity of MEK protein, which is also capable of being regulated by Raf protein. As such, an MEKK protein of the present invention is capable of regulating the activity of MAPK protein and members of the Ets family of transcription factors, such as TCF protein, also referred to as Elk-1 protein.

Referring to Fig. 2, an MEKK protein of the present invention is capable of being activated by a variety of growth factors capable of activating Ras protein. In addition, an MEKK protein is capable of activating JNK protein which is also activated by Ras protein, but is not activated by Raf protein. As such, an MEKK protein of the present invention comprises a protein kinase at a divergence point in a signal transduction pathway initiated by different cell surface receptors. An MEKK protein is also capable of being regulated by TNF protein independent of Raf, thereby indicating an association of MEKK protein to a novel signal transduction pathway which is independent of Ras protein and Raf protein. Thus, an MEKK protein is capable of performing numerous unique functions independent of or by-passing Raf protein in one or more signal transduction pathways. An MEKK protein is capable of regulating the activity of MEK and/or JEK activity. As such, an MEKK protein is capable of regulating the activity of members of a signal transduction pathway that does not substantially include Raf activity. Such members include,

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but are not limited to, JNK, Jun, ATF and Myc protein. In addition, an MEKK protein is capable of regulating the members of a signal transduction pathway that does involve Raf, such members including, but are not limited to, MEK, MAPK and TCF. An MEKK protein of the present invention is thus capable of regulating the apoptosis of a cell independent of significant involvement by Raf protein.

In addition to the numerous functional characteristics of an MEKK protein, an MEKK protein of the present invention comprises numerous unique structural characteristics. For example, in one embodiment, an MEKK protein of the present invention includes at least one of two different structural domains having particular functional characteristics. Such structural domains include an NH₂-terminal regulatory domain that serves to regulate a second structural domain comprising a COOH-terminal protein kinase catalytic domain that is capable of phosphorylating an MEK protein and/or JEK protein.

According to the present invention, an MEKK protein of the present invention includes a full-length MEKK protein, as well as at least a portion of an MEKK protein capable of performing at least one of the functions defined above. The phrase "at least a portion of an MEKK protein" refers to a portion of an MEKK protein encoded by a nucleic acid molecule that is capable of hybridizing, under stringent conditions, with a nucleic acid encoding a full-length MEKK protein of the present invention. Preferred portions of MEKK proteins are useful for regulating apoptosis in a

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cell. Additional preferred portions have activities useful for regulating MEKK kinase activity. Suitable sizes for portions of an MEKK protein of the present invention are as disclosed for MEKK protein homologues of the present invention.

In another embodiment, an MEKK protein of the present invention includes at least a portion of an MEKK protein having molecular weights ranging from about 70 kD to about 250 kD as determined by Tris-glycine SDS-PAGE, preferably using an 8% polyacrylamide SDS gel (SDS-PAGE) and resolved using methods standard in the art. A preferred MEKK protein has a molecular weight ranging from about 75 kD to about 225 kD and even more preferably from about 80 kD to about 200 kD.

In yet another embodiment, an MEKK protein of the present invention comprises at least a portion of an MEKK protein encoded by an mRNA (messenger ribonucleic acid) ranging from about 3.5 kb to about 12.0 kb, more preferably ranging from about 4.0 kb to about 11.0 kb, and even more preferably ranging from about 4.5 kb to about 10.0 kb. Particularly preferred MEKK proteins comprise at least a portion of an MEKK protein encoded by an mRNA having a size ranging from about 4.5 kb to about 5.0 kb, a size ranging from about 6.0 kb to about 6.5 kb, a size of about 7.0 kb, or a size ranging from about 8.0 kb to about 10.0 kb.

In another embodiment, an NH₂-terminal regulatory domain of the present invention includes an NH₂-terminal comprising about 400 amino acids having at least about 10%

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serine and/or threonine residues, more preferably about 400 amino acids having at least about 15% serine and/or threonine residues, and even more preferably about 400 amino acids having at least about 20% serine and/or
5 threonine residues.

A preferred an NH_2 -terminal regulatory domain of the present invention includes an NH_2 -terminal comprising about 360 amino acids having at least about 10% serine and/or threonine residues, more preferably about 360 amino acids
10 having at least about 15% serine and/or threonine residues, and even more preferably about 360 amino acids having at least about 20% serine and/or threonine residues.

Another preferred an NH_2 -terminal regulatory domain of the present invention includes an NH_2 -terminal comprising
15 about 370 amino acids having at least about 10% serine and/or threonine residues, more preferably about 370 amino acids having at least about 15% serine and/or threonine residues, and even more preferably about 370 amino acids having at least about 20% serine and/or threonine residues.

20 In one embodiment, an MEKK protein of the present invention is devoid of SH2 and SH3 domains.

In another embodiment, an MEKK protein of the present invention includes at least a portion of an MEKK protein homologue preferably having at least about 50%, more
25 preferably at least about 75%, and even more preferably at least about 85% amino acid homology (identity within comparable regions) with the kinase catalytic domain of a naturally occurring MEKK protein. Another MEKK protein of

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the present invention also includes at least a portion of an MEKK homologue of the present invention has at least about 10%, more preferably at least about 20%, and even more preferably at least about 30% amino acid homology with the NH₂-terminal regulatory domain of an MEKK protein of a naturally occurring MEKK protein.

The sequences comprising the catalytic domain of an MEKK protein are involved in phosphotransferase activity, and therefore display a relatively conserved amino acid sequence. The NH₂-terminal regulatory domain of an MEKK protein, however, can be substantially divergent. The lack of significant homology between MEKK protein NH₂-terminal regulatory domains is related to the regulation of each of such domains by different upstream regulatory proteins. For example, an MEKK protein can be regulated by the protein Ras, while others can be regulated independent of Ras. In addition, some MEKK proteins can be regulated by the growth factor TNF α , while others cannot. As such, the NH₂-terminal regulatory domain of an MEKK protein provides selectivity for upstream signal transduction regulation, while the catalytic domain provides for MEKK substrate selectivity function.

A preferred MEKK homologue has at least about 50%, more preferably at least about 75% and even more preferably at least about 85% amino acid homology with the kinase catalytic domain of an MEKK protein having an amino acid sequence represented by SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:10. Another preferred MEKK

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homologue has at least about 10%, more preferably at least about 20% and even more preferably at least about 30% amino acid homology with the NH₂-terminal regulatory domain of an MEKK protein having an amino acid sequence represented by
5 SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:10.

In a preferred embodiment, an MEKK protein of the present invention includes at least a portion of an MEKK protein homologue of the present invention that is encoded
10 by a nucleic acid molecule having at least about 50%, more preferably at least about 75%, and even more preferably at least about 85% homology with a nucleic acid molecule encoding the kinase catalytic domain of an MEKK protein. Another preferred MEKK protein homologue is encoded by a
15 nucleic acid molecule having at least about 10%, more preferably at least about 20%, and even more preferably at least about 30% homology with a nucleic acid molecule encoding the NH₂-terminal regulatory domain of an MEKK protein.

20 Still another preferred MEKK homologue is encoded by a nucleic acid molecule having at least about 50%, more preferably at least about 75% and even more preferably at least about 85% amino acid homology with the kinase catalytic domain of an MEKK protein encoded by a nucleic
25 acid sequence represented by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:9. An MEKK homologue also includes those encoded by a nucleic acid molecule having at least about 10%, more preferably at least about

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20% and even more preferably at least about 30% amino acid homology with the NH₂-terminal regulatory domain of an MEKK protein encoded by a nucleic acid sequence represented by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:9.

An MEKK protein of the present invention, referred to here as MEKK 1, includes an MEKK protein having (i.e., including) at least a portion of the nucleic acid and/or an amino acid sequence shown in Table 1 and represented by SEQ ID NO:1 and SEQ ID NO:2, respectively.

Table 1.

	TACACTCCTT GCCACAGTCT GGCAGAAAGA ATCAAACCTC AGAGACTCCT CCGGCCAGTT	60
	GTAGACACTA TCCTTGTCAG GTGTGCAGAT CCAACAGCCG CACGAGTCAG CTGTCCATAT	120
	CTACAGTGT GGAAGCTGTC AAGGGCCAAG CAGGAGAGCT GCGGTTGGG AGAGAAATAC	180
15	TTAAGCTGG GTCCATCGGG GTTGGTGGTG TCGATTACGT CTAAAGTTGT ATCCTTGGAA	240
	ACCAAGCTGA ATCAACAAC TGGCAAGAAC TGCTGGGTGC CCTCTGTCTT ATAGACAGGT	300
	TGCTGTTGGA ATTTCTGCT GAATTCTATC CTCATATTGT CAGTACTGAT GTCTCACAAG	360
	CTGAGCCTGT TGAAATCAGG TACAAGAAGC TGCTCTCCCT CTAAACCTTT GCCTTGCAAT	420
	CCATTGACAA TTCCCACTCG ATGGTTGGCA AGCTCTCTCG GAGGATATAT CTGAGCTCTG	480
20	CCAGG ATG GTG ACC GCA GTG CCC GCT GTG TTT TCC AAG CTG GTA ACC	527
	Met Val Thr Ala Val Pro Ala Val Phe Ser Lys Leu Val Thr	
	1 5 10	
	ATG CTT AAT GCT TCT GGC TCC ACC CAC TTC ACC AGG ATG CGC CGG CGT	575
25	Met Leu Asn Ala Ser Gly Ser Thr His Phe Thr Arg Met Arg Arg Arg	
	15 20 25 30	
	CTG ATG GCT ATC GCG GAT GAG GTA GAA ATT GCC GAG GTC ATC CAG CTG	623
	Leu Met Ala Ile Ala Asp Glu Val Glu Ile Ala Glu Val Ile Gln Leu	
	35 40 45	
30	GGT GTG GAG GAC ACT GTG GAT GGG CAT CAG GAC AGC TTA CAG GCC GTG	671
	Gly Val Glu Asp Thr Val Asp Gly His Gln Asp Ser Leu Gln Ala Val	
	50 55 60	
	GCC CCC ACC AGC TGT CTA GAA AAC AGC TCC CTT GAG CAC ACA GTC CAT	719
	Ala Pro Thr Ser Cys Leu Glu Asn Ser Ser Leu Glu His Thr Val His	
	65 70 75	
35	AGA GAG AAA ACT GGA AAA GGA CTA AGT GCT ACG AGA CTG AGT GCC AGC	767
	Arg Glu Lys Thr Gly Lys Gly Leu Ser Ala Thr Arg Leu Ser Ala Ser	
	80 85 90	
	TCG GAG GAC ATT TCT GAC AGA CTG GCC GGC GTC TCT GTA GGA CTT CCC	815
40	Ser Glu Asp Ile Ser Asp Arg Leu Ala Gly Val Ser Val Gly Leu Pro	
	95 100 105 110	
	AGC TCA ACA ACA ACA GAA CAA CCA AAG CCA GCG GTT CAA ACA AAA GGC	863
	Ser Ser Thr Thr Thr Glu Gln Pro Lys Pro Ala Val Gln Thr Lys Gly	
	115 120 125	
45	AGA CCC CAC AGT CAG TGT TTG AAC TCC TCC CCT TTG TCT CAT GCT CAA	911
	Arg Pro His Ser Gln Cys Leu Asn Ser Ser Pro Leu Ser His Ala Gln	
	130 135 140	

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	TTA ATG TTC CCA GCA CCA TCA GCC CCT TGT TCC TCT GCC CCG TCT GTC Leu Met Phe Pro Ala Pro Ser Ala Pro Cys Ser Ser Ala Pro Ser Val 145 150 155	959
5	CCA GAT ATT TCT AAG CAC AGA CCC CAG GCA TTT GTT CCC TGC AAA ATA Pro Asp Ile Ser Lys His Arg Pro Gln Ala Phe Val Pro Cys Lys Ile 160 165 170	1007
	CCT TCC GCA TCT CCT CAG ACA CAG CGC AAG TTC TCT CTA CAA TTC CAG Pro Ser Ala Ser Pro Gln Thr Gln Arg Lys Phe Ser Leu Gln Phe Gln 175 180 185 190	1055
10	AGG AAC TGC TCT GAA CAC CGA GAC TCA GAC CAG CTC TCC CCA GTC TTC Arg Asn Cys Ser Glu His Arg Asp Ser Asp Gln Leu Ser Pro Val Phe 195 200 205	1103
15	ACT CAG TCA AGA CCC CCA CCC TCC AGT AAC ATA CAC AGG CCA AAG CCA Thr Gln Ser Arg Pro Pro Pro Ser Ser Asn Ile His Arg Pro Lys Pro 210 215 220	1151
	TCC CGA CCC GTT CCG GGC AGT ACA AGC AAA CTA GGG GAC GCC ACA AAA Ser Arg Pro Val Pro Gly Ser Thr Ser Lys Leu Gly Asp Ala Thr Lys 225 230 235	1199
20	AGT AGC ATG ACA CTT GAT CTG GGC AGT GCT TCC AGG TGT GAC GAC AGC Ser Ser Met Thr Leu Asp Leu Gly Ser Ala Ser Arg Cys Asp Asp Ser 240 245 250	1247
	TTT GGC GGC GGC GGC AAC AGT GGC AAC GCC GTC ATA CCC AGC GAC GAG Phe Gly Gly Gly Gly Asn Ser Gly Asn Ala Val Ile Pro Ser Asp Glu 255 260 265 270	1295
25	ACA GTG TTC ACG CCG GTG GAG GAC AAG TGC AGG TTA GAT GTG AAC ACC Thr Val Phe Thr Pro Val Glu Asp Lys Cys Arg Leu Asp Val Asn Thr 275 280 285	1343
30	GAG CTC AAC TCC AGC ATC GAG GAC CTT CTT GAA GCA TCC ATG CCT TCA Glu Leu Asn Ser Ser Ile Glu Asp Leu Leu Glu Ala Ser Met Pro Ser 290 295 300	1391
	AGT GAC ACG ACA GTC ACT TTC AAG TCC GAA GTC GCC GTC CTC TCT CCG Ser Asp Thr Thr Val Thr Phe Lys Ser Glu Val Ala Val Leu Ser Pro 305 310 315	1439
35	GAA AAG GCC GAA AAT GAC GAC ACC TAC AAA GAC GAC GTC AAT CAT AAT Glu Lys Ala Glu Asn Asp Asp Thr Tyr Lys Asp Asp Val Asn His Asn 320 325 330	1487
	CAA AAG TGC AAA GAA AAG ATG GAA GCT GAA GAG GAG GAG GCT TTA GCG Gln Lys Cys Lys Glu Lys Met Glu Ala Glu Glu Glu Ala Leu Ala 335 340 345 350	1535
40	ATC GCC ATG GCG ATG TCA GCG TCT CAG GAT GCC CTC CCC ATC GTC CCT Ile Ala Met Ala Met Ser Ala Ser Gln Asp Ala Leu Pro Ile Val Pro 355 360 365	1583
45	CAG CTG CAG GTG GAA AAT GGA GAA GAT ATT ATC ATC ATT CAG CAG GAC Gln Leu Gln Val Glu Asn Gly Glu Asp Ile Ile Ile Ile Gln Gln Asp 370 375 380	1631
	ACA CCA GAA ACT CTT CCA GGA CAT ACC AAA GCG AAA CAG CCT TAC AGA Thr Pro Glu Thr Leu Pro Gly His Thr Lys Ala Lys Gln Pro Tyr Arg 385 390 395	1679
50	GAA GAC GCT GAG TGG CTG AAA GGC CAG CAG ATA GGC CTC GGA GCA TTT Glu Asp Ala Glu Trp Leu Lys Glu Lys Gln Gln Ile Gly Leu Gly Ala Phe 400 405 410	1727
	TCT TCC TGT TAC CAA GCA CAG GAT GTG GGG ACT GGG ACT TTA ATG GCT Ser Ser Cys Tyr Gln Ala Gln Asp Val Gly Thr Gly Thr Leu Met Ala 415 420 425 430	1775
55	GTG AAA CAG GTG ACG TAC GTC AGA AAC ACA TCC TCC GAG CAG GAG GAG Val Lys Gln Val Thr Tyr Val Arg Asn Thr Ser Ser Glu Gln Glu Glu 435 440 445 450	1823

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	435	440	445	
	GTG GTG GAA GCG TTG AGG GAA GAG ATC CGG ATG ATG GGT CAC CTC AAC Val Val Glu Ala Leu Arg Glu Glu Ile Arg Met Met Gly His Leu Asn 450 455 460	1871		
5	CAT CCA AAC ATC ATC CGG ATG CTG GGG GCC ACG TGC GAG AAG AGC AAC His Pro Asn Ile Ile Arg Met Leu Gly Ala Thr Cys Glu Lys Ser Asn 465 470 475	1919		
10	TAC AAC CTC TTC ATT GAG TGG ATG GCG GGA GGA TCT GTG GCT CAC CTC Tyr Asn Leu Phe Ile Glu Trp Met Ala Gly Gly Ser Val Ala His Leu 480 485 490	1967		
	TTG AGT AAA TAC GGA GCT TTC AAG GAG TCA GTC GTC ATT AAC TAC ACT Leu Ser Lys Tyr Gly Ala Phe Lys Glu Ser Val Val Ile Asn Tyr Thr 495 500 505 510	2015		
15	GAG CAG TTA CTG CGT GGC CTT TCC TAT CTC CAC GAG AAC CAG ATC ATT Glu Gln Leu Leu Arg Gly Leu Ser Tyr Leu His Glu Asn Gln Ile Ile 515 520 525	2063		
	CAC AGA GAC GTC AAA GGT GCC AAC CTG CTC ATT GAC AGC ACC GGT CAG His Arg Asp Val Lys Gly Ala Asn Leu Leu Ile Asp Ser Thr Gly Gln 530 535 540	2111		
20	AGG CTG AGA ATT GCA GAC TTT GGA GCT GCT GCC AGG TTG GCA TCA AAA Arg Leu Arg Ile Ala Asp Phe Gly Ala Ala Ala Arg Leu Ala Ser Lys 545 550 555	2159		
25	GGA ACC GGT GCA GGA GAG TTC CAG GGA CAG TTA CTG GGG ACA ATT GCA Gly Thr Gly Ala Gly Glu Phe Gln Gly Gln Leu Leu Gly Thr Ile Ala 560 565 570	2207		
	TTC ATG GCG CCT GAG GTC CTA AGA GGT CAG CAG TAT GGT AGG AGC TGT Phe Met Ala Pro Glu Val Leu Arg Gly Gln Gln Tyr Gly Arg Ser Cys 575 580 585 590	2255		
30	GAT GTA TGG AGT GTT GGC TGC GCC ATT ATA GAA ATG GCT TGT GCA AAA Asp Val Trp Ser Val Gly Cys Ala Ile Ile Glu Met Ala Cys Ala Lys 595 600 605	2303		
	CCA CCT TGG AAT GCA GAA AAA CAC TCC AAT CAT CTC GCC TTG ATA TTT Pro Pro Trp Asn Ala Glu Lys His Ser Asn His Leu Ala Leu Ile Phe 610 615 620	2351		
35	AAG ATT GCT AGC GCA ACT ACT GCA CCG TCC ATC CCG TCA CAC CTG TCC Lys Ile Ala Ser Ala Thr Thr Ala Pro Ser Ile Pro Ser His Leu Ser 625 630 635	2399		
40	CCG GGT CTG CGC GAC GTG GCC GTG CGC TGC TTA GAA CTT CAG CCT CAG Pro Gly Leu Arg Asp Val Ala Val Arg Cys Leu Glu Leu Gln Pro Gln 640 645 650	2447		
	GAC CGG CCT CCG TCC AGA GAG CTG CTG AAA CAT CCG GTC TTC CGT ACC Asp Arg Pro Pro Ser Arg Glu Leu Leu Lys His Pro Val Phe Arg Thr 655 660 665 670	2495		
45	ACG TGG TAGTTAATTG TTCAGATCAG CTCTAATGGA GACAGGATAT CGAACCGGGA Thr Trp	2551		
50	GAGAGAAAAG AGAACTTGTG GGGGACCATG CCGCTAACCG CAGCCCTCAC GCCACTGAAC AGCCAGAAAC GGGGCCAGCG GGGAAACCGTA CCTAAGCATG TGATTGACAA ATCATGACCT GTACCTAAGC TCGATATGCA GACATCTACA GCTCGTGCAG GAACTGCACA CCGTGCCTTT CACAGGACTG GCTCTGGGGG ACCAGGAAGG CGATGGAGTT TGCATGACTA AAGAACAGAA GCATAAAATTT ATTTTGGAG CACTTTTTC A GCTAATCAGT ATTACCATGT ACATCAACAT GCCCGCCACA TTTCAAACTC AGACTGTCCC AGATGTCAAG ATCCACTGTG TTTGAGTTTG TTTGCAGTTC CCTCAGCTTG CTGGTAATTG TGGTGTTTTG TTTTCGATGC AAATGTGATG TAATATTCTT ATTTTCTTGG GATCAAAGCT GGACTGAAAA TTGTAAGTGT TAATTATTTT 55 TGTGTTTTTA ATGTTATTGG GTACTCGAAT TGTAATAAAC GTCTACTGCT GTTTATTCCA GTTTCTACTA CCTCAGGTGT CCTATAGATT TTTCTTCTAC CAAAGTTCAC TCTCAGAAAG AAATTCACG TGCTGTGTGA CTATGACTCC TAAGACTTCC AGGGCTTAAG GGCTAACTCC TATTAGCACC TTACTATGTA AGCAAATGCT ACAAAAAAAA AAAAAAAA	2611 2671 2731 2791 2851 2911 2971 3031 3091 3151 3211 3260		

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An MEKK protein of the present invention, referred to here as MEKK 2, includes an MEKK protein having at least a portion of the nucleic acid and/or amino acid sequence shown in Table 2 and represented by SEQ ID NO:3 and SEQ ID NO:4, respectively.

Table 2.

	GGTGGCGGCC GCTCTAGAAC TAGTGGATCC CCCGGGCTGC AGGAATTCGG CACGAGGGAC	60
	GATCCAGCGG CAGAGTCGCC GCTTCCGCTT CGCTGCTTCT CCGGTCGGCG ACGCGGGCCC	120
	GGGGCTTCCT TTTCATCGGC CCAGCTTATT CCGCGGGCCC CCGGGCTGCA GCTACCCAGA	180
10	AGCGGCGAAG AGGCCCTGGG CTGCGCGCCC GCTGTCCCAT GTGAAGCAGG TTGGCCCTGG	240
	TCCCCGGCCC GTGCCCGGTT GTCTGCGGCC CTTCAGGCCCT CAGGGACCCC CGCGAGGCGC	300
	TGCTCCTGGG GGGCGCGGTG ACAGGCCGTG CCGGGGCGGA GGGGCCAGCT CGGTGGCCTC	360
	CTCTCGGCC TCAGCTCCGC GATCCCGCC AGCGGCCGGG CAATAAGAA TGTGTATGGG	420
15	AGAACCATTY TCCTAATTTT CAAATTATTG AGCTGGTCGC GCATA ATG GAT GAT	474
	Met Asp Asp	
	1	
	CAG CAA GCT TTG AAT TCA ATC ATG CAA GAT TTG GCT GTC CTT CAT AAG	522
	Gln Gln Ala Leu Asn Ser Ile Met Gln Asp Leu Ala Val Leu His Lys	
	5 10 15	
20	CCA GTC GGC CAG CAT TAT CTT TAC AAG AAA CCA GGA AAG CAA AAC CTT	570
	Pro Val Gly Gln His Tyr Leu Tyr Lys Lys Pro Gly Lys Gln Asn Leu	
	20 25 30 35	
	CAT CAC CAA AAA AAC AGA ATG ATG TTC GAG TCA AAT TTG AAC ATA GAG	618
25	His His Gln Lys Asn Arg Met Met Phe Glu Ser Asn Leu Asn Ile Glu	
	40 45 50	
	GAG GAA AAA AGG ATC CTG CAG GTT ACT AGA CCA GTT AAA CTA GAA GAC	666
	Glu Glu Lys Arg Ile Leu Gln Val Thr Arg Pro Val Lys Leu Glu Asp	
	55 60 65	
30	CTG AGA TCT AAG TCT AAG ATC GCC TTT GGG CAG TCT ATG GAT CTA CAC	714
	Leu Arg Ser Lys Ser Lys Ile Ala Phe Gly Gln Ser Met Asp Leu His	
	70 75 80	
	TAT ACC AAC AAT GAG TTG GTA ATT CCG TTA ACT ACC CAA GAT GAC TTG	762
	Tyr Thr Asn Asn Glu Leu Val Ile Pro Leu Thr Thr Gln Asp Asp Leu	
	85 90 95	
35	GAC AAA GCT GTG GAA CTG CTG GAT CGC AGT ATT CAC ATG AAG AGT CTC	810
	Asp Lys Ala Val Glu Leu Leu Asp Arg Ser Ile His Met Lys Ser Leu	
	100 105 110 115	
	AAG ATA TTA CTT GTA GTA AAT GGG AGT ACA CAG GCT ACT AAT TTA GAA	858
40	Lys Ile Leu Leu Val Val Asn Gly Ser Thr Gln Ala Thr Asn Leu Glu	
	120 125 130	
	CCA TCA CCG TCA CCA GAA GAT TTG AAT AAT ACA CCA CTT GGT GCA GAG	906
	Pro Ser Pro Ser Pro Glu Asp Leu Asn Asn Thr Pro Leu Gly Ala Glu	
	135 140 145	
45	AGG AAA AAG CGG CTA TCT GTA GTA GGT CCC CCT AAT AGG GAT AGA AGT	954
	Arg Lys Lys Arg Leu Ser Val Val Gly Pro Pro Asn Arg Asp Arg Ser	
	150 155 160	
	TCC CCT CCT CCA GGA TAC ATT CCA GAC ATA CTA CAC CAG ATT GCC CGG	1002
	Ser Pro Pro Pro Gly Tyr Ile Pro Asp Ile Leu His Gln Ile Ala Arg	
	165 170 175	

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	AAT GGG TCA TTC ACT AGC ATC AAC AGT GAA GGA GAG TTC ATT CCA GAG Asn Gly Ser Phe Thr Ser Ile Asn Ser Glu Gly Glu Phe Ile Pro Glu 180 185 190 195	1050
5	AGC ATG GAC CAA ATG CTG GAT CCA TTG TCT TTA AGC AGC CCT GAA AAT Ser Met Asp Gln Met Leu Asp Pro Leu Ser Leu Ser Ser Pro Glu Asn 200 205 210	1098
	TCT GGC TCA GGA AGC TGT CCG TCA CTT GAT AGT CCT TTG GAT GGA GAA Ser Gly Ser Gly Ser Cys Pro Ser Leu Asp Ser Pro Leu Asp Gly Glu 215 220 225	1146
10	AGC TAC CCA AAA TCA CGG ATG CCT AGG GCA CAG AGC TAC CCA GAT AAT Ser Tyr Pro Lys Ser Arg Met Pro Arg Ala Gln Ser Tyr Pro Asp Asn 230 235 240	1194
15	CAT CAG GAG TTT ACA GAC TAT GAT AAC CCC ATT TTT GAG AAA TTT GGA His Gln Glu Phe Thr Asp Tyr Asp Asn Pro Ile Phe Glu Lys Phe Gly 245 250 255	1242
	AAA GGA GGA ACA TAT CCA AGA AGG TAC CAC GTT TCC TAT CAT CAC CAG Lys Gly Gly Thr Tyr Pro Arg Arg Tyr His Val Ser Tyr His His Glu 260 265 270 275	1290
20	GAG TAT AAT GAC GGT CGG AAG ACT TTT CCA AGA GCT AGA AGG ACC CAG Glu Tyr Asn Asp Gly Arg Lys Thr Phe Pro Arg Ala Arg Arg Thr Gln 280 285 290	1338
	GGC ACC AGT TTC CGG TCT CCT GTG AGC TTC AGT CCT ACT GAT CAC TCC Gly Thr Ser Phe Arg Ser Pro Val Ser Phe Ser Pro Thr Asp His Ser 295 300 305	1386
25	TTA AGC ACT AGT AGT GGA AGC AGT GTC TTT ACC CCA GAG TAT GAC GAC Leu Ser Thr Ser Ser Gly Ser Ser Val Phe Thr Pro Glu Tyr Asp Asp 310 315 320	1434
30	AGT CGA ATA AGA AGA CGG GGG AGT GAC ATA GAC AAT CCT ACT TTG ACT Ser Arg Ile Arg Arg Arg Gly Ser Asp Ile Asp Asn Pro Thr Leu Thr 325 330 335	1482
	GTC ACA GAC ATC AGC CCA CCC AGC CGT TCA CCT CGA GCT CCG ACC AAC Val Thr Asp Ile Ser Pro Pro Ser Arg Ser Pro Arg Ala Pro Thr Asn 340 345 350 355	1530
35	TGG AGA CTG GGC AAG CTG CTT GGC CAA GGA GCT TTT GGT AGG GTC TAC Trp Arg Leu Gly Lys Leu Leu Gly Gln Gly Ala Phe Gly Arg Val Tyr 360 365 370	1578
	CTC TGC TAT GAT GTT GAT ACC GGA AGA GAG CTG GCT GTT AAG CAA GTT Leu Cys Tyr Asp Val Asp Thr Gly Arg Glu Leu Ala Val Lys Gln Val 375 380 385	1626
40	CAG TTT AAC CCT GAG AGC CCA GAG ACC AGC AAG GAA GTA AAT GCA CTT Gln Phe Asn Pro Glu Ser Pro Glu Thr Ser Lys Glu Val Asn Ala Leu 390 395 400	1674
45	GAG TGT GAA ATT CAG TTG TTG AAA AAC TTG TTG CAT GAG CGA ATT GTT Glu Cys Glu Ile Gln Leu Leu Lys Asn Leu Leu His Glu Arg Ile Val 405 410 415	1722
	CAG TAT TAT GGC TGT TTG AGG GAT CCT CAG GAG AAA ACA CTT TCC ATC Gln Tyr Tyr Gly Cys Leu Arg Asp Pro Gln Glu Lys Thr Leu Ser Ile 420 425 430 435	1770
50	TTT ATG GAG CTC TCG CCA GGG GGT TCA ATT AAG GAC CAA CTA AAA GCC Phe Met Glu Leu Ser Pro Gly Gly Ser Ile Lys Asp Gln Leu Lys Ala 440 445 450	1818
	TAC GGA GCT CTT ACT GAG AAC GTG ACG AGG AAG TAC ACC CGT CAG ATT Tyr Gly Ala Leu Thr Glu Asn Val Thr Arg Lys Tyr Thr Arg Gln Ile 455 460 465	1866

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	CTG GAG GGG GTC CAT TAT TTG CAT AGT AAT ATG ATT GTC CAT AGA GAT Leu Glu Gly Val His Tyr Leu His Ser Asn Met Ile Val His Arg Asp 470 475 480	1914
5	ATC AAA GGA GCA AAT ATC TTA AGG GAT TCC ACA GGC AAT ATC AAG TTA Ile Lys Gly Ala Asn Ile Leu Arg Asp Ser Thr Gly Asn Ile Lys Leu 485 490 495	1962
	GGA GAC TTT GGG GCT AGT AAA CGG CTT CAG ACC ATC TGT CTC TCA GGC Gly Asp Phe Gly Ala Ser Lys Arg Leu Gln Thr Ile Cys Leu Ser Gly 500 505 510 515	2010
10	ACA GGA ATG AAG TCT GTC ACA GGC ACG CCA TAC TGG ATG AGT CCT GAG Thr Gly Met Lys Ser Val Thr Gly Thr Pro Tyr Trp Met Ser Pro Glu 520 525 530	2058
15	GTC ATC AGT GGA GAA GGC TAT GGA AGA AAA GCA GAC ATC TGG AGT GTA Val Ile Ser Gly Glu Gly Tyr Gly Arg Lys Ala Asp Ile Trp Ser Val 535 540 545	2106
	GCA TGT ACT GTG GTA GAA ATG CTA ACT GAA AAG CCA CCT TGG GCT GAA Ala Cys Thr Val Val Glu Met Leu Thr Glu Lys Pro Pro Trp Ala Glu 550 555 560	2154
20	TTT GAA GCA ATG GCT GCC ATC TTT AAG ATC GCC ACT CAG CCA ACG AAC Phe Glu Ala Met Ala Ala Ile Phe Lys Ile Ala Thr Gln Pro Thr Asn 565 570 575	2202
	CCA AAG CTG CCA CCT CAT GTC TCA GAC TAT ACT CGG GAC TTC CTC AAA Pro Lys Leu Pro Pro His Val Ser Asp Tyr Thr Arg Asp Phe Leu Lys 580 585 590 595	2250
25	CGG ATT TTT GTA GAG GCC AAA CTT CGA CCT TCA GCG GAG GAG CTC TTG Arg Ile Phe Val Glu Ala Lys Leu Arg Pro Ser Ala Glu Glu Leu Leu 600 605 610	2298
30	CGG CAC ATG TTT GTG CAT TAT CAC TAGCAGCGGC GGCTTCGGTC CTCACCAGC Arg His Met Phe Val His Tyr His 615 620	2352
	TCATCCTCG CGGCCACCTT CTCTCTACT GCACTTTCCT TTTTATAAA AAAGAGAGAT GGGGAGAAAA AGACAAGAGG GAAATATTT CTCTTGATTC TTGGTTAAAT TTGTTAATA ATAATAGTAA ACTAAAAAAA AAAAAAAAAA A	2412 2472 2503

An MEKK protein of the present invention, referred to
 35 here as MEKK 3, includes an MEKK protein having at least a
 portion of the nucleic acid and/or amino acid sequence
 shown in Table 3 and represented by SEQ ID NO:5 and SEQ ID
 NO:6, respectively.

Table 3.

40	AGGGAACAAA AGCTGGAGCT CCACCGCGGT GCGGCGCGCT CTAGAACTAG TGGATCCCCC GGGCTGCAGG AATTCGGCAC GAGGAACAGT GGCCGGTCGG AGCGTCTTCT GGACTTCAGG ACTCGCAGGC GGCCCGGTCG AGTGGCGCCG CCGAGGCCGG GTTGGGCCGA GCCTGGGAGC GCCGGGGATG TAGCGGGCCA ACCTGCTCAT GCCACAGCGC CCGGCCGCGG CCGAGCCGGA GCCTGGGGAG GCGCGGGGG CCCCAGCGC AGCCACGGC CCCCAGCGG AGCCAGGCCC GTGCGCGTCC CCGCCGCGCG CTCCCCGCGC ATGACGCCCC GGCTGCGGAG GTGACACTTC	60 120 180 240 300 360
45	TGGGCTGTAG TCGCCACCGC CGCCTCCGCC ATCGCCACC ATG GAT GAA CAA GAG Met Asp Glu Gln Glu 1 5	414

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	GCA TTA GAC TCG ATC ATG AAG GAC CTG GTG GCC CTC CAG ATG AGC CGA Ala Leu Asp Ser Ile Met Lys Asp Leu Val Ala Leu Gln Met Ser Arg 10 15 20	462
5	CGA ACC CGG TTG TCT GGA TAT GAG ACC ATG AAG AAT AAG GAC ACA GGT Arg Thr Arg Leu Ser Gly Tyr Glu Thr Met Lys Asn Lys Asp Thr Gly 25 30 35	510
	CAC CCA AAC AGG CAG AGT GAC GTC AGA ATC AAG TTT GAA CAC AAT GGG His Pro Asn Arg Gln Ser Asp Val Arg Ile Lys Phe Glu His Asn Gly 40 45 50	558
10	GAG AGA CGA ATT ATA GCA TTC AGC CGG CCT GTG AGA TAC GAA GAT GTG Glu Arg Arg Ile Ile Ala Phe Ser Arg Pro Val Arg Tyr Glu Asp Val 55 60 65	606
15	GAG CAC AAG GTG ACA ACA GTC TTT GGG CAG CCT CTT GAT TTG CAT TAT Glu His Lys Val Thr Thr Val Phe Gly Gln Pro Leu Asp Leu His Tyr 70 75 80 85	654
	ATG AAT AAT GAG CTC TCC ATC CTG TTG AAA AAC CAA GAT GAT CTC GAT Met Asn Asn Glu Leu Ser Ile Leu Leu Lys Asn Gln Asp Asp Leu Asp 90 95 100	702
20	AAA GCC ATT GAC ATT TTG GAT AGA AGC TCA AGT ATG AAA AGC CTT AGG Lys Ala Ile Asp Ile Leu Asp Arg Ser Ser Ser Met Lys Ser Leu Arg 105 110 115	750
	ATA CTA CTG TTA TCC CAA GAC AGA AAC CAT ACT AGT TCC TCT CCC CAC Ile Leu Leu Leu Ser Gln Asp Arg Asn His Thr Ser Ser Ser Pro His 120 125 130	798
25	TCT GGA GTG TCC AGG CAG GTT CGG ATC AAG CCT TCC CAG TCT GCA GGG Ser Gly Val Ser Arg Gln Val Arg Ile Lys Pro Ser Gln Ser Ala Gly 135 140 145	846
30	GAT ATA AAT ACC ATC TAC CAA GCT CCT GAG CCC AGA AGC AGG CAC CTG Asp Ile Asn Thr Ile Tyr Gln Ala Pro Glu Pro Arg Ser Arg His Leu 150 155 160 165	894
	TCT GTC AGC TCC CAG AAC CCT GGC CGA AGC TCT CCT CCC CCG GGA TAT Ser Val Ser Ser Gln Asn Pro Gly Arg Ser Ser Pro Pro Pro Gly Tyr 170 175 180	942
35	GTA CCT GAG CGA CAA CAG CAC ATT GCC CGG CAA GGA TCC TAT ACG AGC Val Pro Glu Arg Gln Gln His Ile Ala Arg Gln Gly Ser Tyr Thr Ser 185 190 195	990
	ATC AAC AGC GAA GGT GAA TTC ATC CCA GAG ACC AGC GAA CAG TGT ATG Ile Asn Ser Glu Gly Glu Phe Ile Pro Glu Thr Ser Glu Gln Cys Met 200 205 210	1038
40	CTA GAT CCC CTC AGC AGT GCC GAA AAT TCC TTG TCA GGA AGC TGC CAA Leu Asp Pro Leu Ser Ser Ala Glu Asn Ser Leu Ser Gly Ser Cys Gln 215 220 225	1086
45	TCC TTG GAC AGG TCA GCA GAC AGC CCA TCC TTC AGG AAA TCA CAA ATG Ser Leu Asp Arg Ser Ala Asp Ser Pro Ser Phe Arg Lys Ser Gln Met 230 235 240 245	1134
	TCC CGA GCC CGG AGC TTC CCA GAC AAC AGA AAG GAA TGC TCA GAT CGG Ser Arg Ala Arg Ser Phe Pro Asp Asn Arg Lys Glu Cys Ser Asp Arg 250 255 260	1182
50	GAG ACC CAG CTC TAT GAT AAA GGT GTC AAA GGT GGA ACC TAT CCC AGG Glu Thr Gln Leu Tyr Asp Lys Gly Val Lys Gly Gly Thr Tyr Pro Arg 265 270 275	1230
	CGC TAC CAT GTG TCT GTG CAT CAC AAA GAC TAC AAT GAT GGC AGA AGA Arg Tyr His Val Ser Val His His Lys Asp Tyr Asn Asp Gly Arg Arg 280 285 290	1278

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	ACA TTT CCC CGA ATA CGA CGG CAT CAA GGC AAC CTA TTC ACT CTG GTG Thr Phe Pro Arg Ile Arg Arg His Gln Gly Asn Leu Phe Thr Leu Val 295 300 305	1326
5	CCC TCA AGT CGC TCC TTG AGC ACA AAT GGC GAG AAC ATG GGT GTA GCT Pro Ser Ser Arg Ser Leu Ser Thr Asn Gly Glu Asn Met Gly Val Ala 310 315 320 325	1374
	GTG CAA TAC CTG GAC CCC CGT GGG CGC CTA CGG AGT GCA GAC AGT GAG Val Gln Tyr Leu Asp Pro Arg Gly Arg Leu Arg Ser Ala Asp Ser Glu 330 335 340	1422
10	AAT GCC CTC ACT GTG CAG GAA AGG AAT GTG CCA ACC AAA TCT CCT AGT Asn Ala Leu Thr Val Gln Glu Arg Asn Val Pro Thr Lys Ser Pro Ser 345 350 355	1470
15	GCT CCC ATC AAT TGG CGT CGG GGG AAG CTC CTG GGT CAA GGT GCC TTC Ala Pro Ile Asn Trp Arg Arg Gly Lys Leu Leu Gly Gln Gly Ala Phe 360 365 370	1518
	GGC AGG GTC TAC TTG TGC TAT GAT GTG GAC ACA GGA CGT GAA CTT GCT Gly Arg Val Tyr Leu Cys Tyr Asp Val Asp Thr Gly Arg Glu Leu Ala 375 380 385	1566
20	TCT AAG CAG GTC CAG TTT GAC CCA GAT AGT CCT GAG ACA AGC AAG GAG Ser Lys Gln Val Gln Phe Asp Pro Asp Ser Pro Glu Thr Ser Lys Glu 390 395 400 405	1614
	GTG AGT GCT CTG GAG TGT GAG ATC CAG TTG CTG AAG AAC CTG CAG CAT Val Ser Ala Leu Glu Cys Glu Ile Gln Leu Leu Lys Asn Leu Gln His 410 415 420	1662
25	GAG CGC ATT GTG CAG TAC TAC GGC TGC CTG CGG GAC CGT GCT GAG AAG Glu Arg Ile Val Gln Tyr Tyr Gly Cys Leu Arg Asp Arg Ala Glu Lys 425 430 435	1710
30	ATC CTC ACC ATC TTT ATG GAG TAT ATG CCA GGG GGC TCT GTA AAA GAC Ile Leu Thr Ile Phe Met Glu Tyr Met Pro Gly Gly Ser Val Lys Asp 440 445 450	1758
	CAG TTG AAG GCC TAC GGA GCT CTG ACA GAG AGT GTG ACC CGC AAG TAC Gln Leu Lys Ala Tyr Gly Ala Leu Thr Glu Ser Val Thr Arg Lys Tyr 455 460 465	1806
35	ACC CGG CAG ATT CTG GAG GGC ATG TCA TAC CTG CAC AGC AAC ATG ATT Thr Arg Gln Ile Leu Glu Gly Met Ser Tyr Leu His Ser Asn Met Ile 470 475 480 485	1854
	GTG CAT CGG GAC ATC AAG GGA GCC AAT ATC CTC CGA GAC TCA GCT GGG Val His Arg Asp Ile Lys Gly Ala Asn Ile Leu Arg Asp Ser Ala Gly 490 495 500	1902
40	AAT GTG AAG CTT GGG GAT TTT GGG GCC AGC AAA CGC CTA CAG ACC ATC Asn Val Lys Leu Gly Asp Phe Gly Ala Ser Lys Arg Leu Gln Thr Ile 505 510 515	1950
45	TGC ATG TCA GGG ACA GGC ATT CGC TCT GTC ACT GGC ACA CCC TAC TGG Cys Met Ser Gly Thr Gly Ile Arg Ser Val Thr Gly Thr Pro Tyr Trp 520 525 530	1998
	ATG AGT CCT GAA GTC ATC AGT GGC GAG GGC TAT GGA AGA AAG GCA GAC Met Ser Pro Glu Val Ile Ser Gly Glu Gly Tyr Gly Arg Lys Ala Asp 535 540 545	2046
50	GTG TGG AGC CTG GGC TGT ACT GTG GTG GAA ATG CTG ACA GAG AAA CCA Val Trp Ser Leu Gly Cys Thr Val Val Glu Met Leu Thr Glu Lys Pro 550 555 560 565	2094
	CCT TGG GCA GAG TAT GAA GCT ATG GCT GCC ATT TTC AAG ATT GCC ACC Pro Trp Ala Glu Tyr Glu Ala Met Ala Ala Ile Phe Lys Ile Ala Thr 570 575 580	2142

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	CAG CCT ACC AAT CDT CAG CTG CCC TCT CAC ATC TCA GAA CAC GGC AGG	2190
	Gln Pro Thr Asn Pro Gln Leu Pro Ser His Ile Ser Glu His Gly Arg	
	585 590 595	
5	GAC TTC CTG AGG CGC ATA TTT GTG GAA GCT CGT CAG AGA CCC TCA GCT	2238
	Asp Phe Leu Arg Arg Ile Phe Val Glu Ala Arg Gln Arg Pro Ser Ala	
	600 605 610	
	GAG GAG CTG CTC ACA CAC CAC TTT GCA CAG CTA GTG TAC TGAGCTCTCA	2287
	Glu Glu Leu Leu Thr His His Phe Ala Gln Leu Val Tyr	
	615 620 625	
10	AGGCTATCAG GGTGCCAGCT GCCACCTGCT GAGCAGGCAA GGGGCTGCTG TCAGGCTCAG	2347
	TGAAGTTGCT GCTTCTTCCA GGCAAGGCTA TGACCAGTGG AGCATCGGTC CAGCCATTGT	2407
	TTGTCTGTGC CCCATCTGCC ACTGGGACTC AAAGCCAGGA TGGGATAGCT CTGGCATCAA	2467
	GACTGGGAGC TCCAGCCTGT AAGACCCAAG AGCTTTAGCA CCTTAAGCTC AGTATGGCGG	2527
15	GAAGGGCTGG AAACAGTATG CAAGACTGCC ATGGGTCCTG CCTACCCTCA GATGTGTCT	2587
	AACTAGTCAG ACAGCACTGA AGTCAAGAGG GACTGGGGCA CAGGAGGTCC TCAAGGGTAT	2647
	GAATAGTGT ACTTCATTCA GAGTGTACT TTGTTTCTCT CCAATGTTT GGAGACCACC	2707
	AGCCTGTCTC TGGGCTGCAA GCCTGAGGTA AAGCCCAGCA TCCCCAGGCC AACAGAAGGT	2767
	AGAGGTTTGG GCTACCCAC TATAGCTTCC AGGTATTCGG TGTCAGTCTT GTCTTACCAA	2827
	AGATGAATGA AGCAAATGTT ACACCTGCCTT ATTCTGGGAA GGAGGAGCTA CTCGGTAAG	2887
20	CAGGGCCTGA GAGATGGAGC TGCCTCCAGA AACTGGGGAG ACCCAGTCTT GTCAATGCAA	2947
	TTGTCTCTGT TTTACAAATT GGAGTCACTC TTATGCTGTT CCCAGTTTAA AACTGGGAGA	3007
	CTTTGCCCTC TGAGCTCTGG AGACCCATGT GGGCTTAGGC TTGGACTGGA TGAAGAGCT	3067
	GATGGCTCT GCCCTGGCC TG	3089

An MEKK protein of the present invention can also
 25 include an MEKK protein having at least a portion of the
 nucleic acid and/or amino acid sequence shown in Table 4
 and represented by SEQ ID NO:7 and SEQ ID NO:8,
 respectively, and is referred to as MEKK 4.

Table 4.

30	AATTCGGCAC GAGAACCTAT CAGACATTGG CTGGCCAGTG TTTGAAATCC CCTCCCTCG	60
	GCCGTCCAAG GGCTACGAGC CAGAGGACGA GGTGAGGAC ACGGAGGTTG AGCTGAGGGA	120
	GCTGGAGAGC GGGACGGAGG AGAGTGACGA GGAGCCAACC CCCAGTCCGA GGGTGCCAGA	180
	GCTCAGGCTG TCCACAGACA CCATCTTGGA CAGTCGCTCC CAGGGCTGCG TCTCCAGGAA	240
	GCTGGAGAGG CTCGAGTCAG AGGAAGATTC CATAGGCTGG GGGACAGCGG ACTGTGGCCC	300
35	TGAAGCCAGC AGGCATTGTT TGACTTCTAT CTATAGACCA TTCGTGGACA AAGCACTGAA	360
	GCAAATGGGG CTAAGAAAGT TAATTTTACG ACTTCATAAG CTTATGAATG GGTCTTGCA	420
	AAGAGCTCGT GTAGCTCTGG TGAAGGACGA CCGTCAGTGG AGTTCTCTGA CTTTCCAGGT	480
	CCCATGTGGG GCTCGGATTA TGTGCAGTTG TCGGGAACAC CTCCTTCCTC AGAGCAGAAG	540
	TGTAGCGCTG TGTCTTGGGA AGAAGTGAAG GCCATGGACC TGCCTTCCTT TGAGCCCGCC	600
40	TTCTGTGTC TCTGTGGGT CCTGCTGAAC GTGATCCAGC AGTGCCCTGAA GGTGCGGCTG	660
	GAACAGAGGC TGCCGGGGAG CCTTCCCTCT TGAGTATCAA ACAGCTAGTG CGAGAGTGTA	720
	AAGAGGTCCT AAAGGGCGGG CTCCTG ATG AAG CAG TAT TAC CAG TTC ATG CTG	773
	Met Lys Gln Tyr Tyr Gln Phe Met Leu	
	1 5	
45	CAG GAG GTC CTG GGC GGA CTG GAG AAG ACC GAC TGC AAC ATG GAT GCC	821
	Gln Glu Val Leu Gly Gly Leu Glu Lys Thr Asp Cys Asn Met Asp Ala	
	10 15 20 25	
	TTT GAG GAG GAC CTG CAG AAG ATG CTG ATG GTG TAT TTT GAT TAC ATG	869
	Phe Glu Glu Asp Leu Gln Lys Met Leu Met Val Tyr Phe Asp Tyr Met	
50	30 35 40	
	AGA AGC TGG ATC CAA ATG CTA CAG CAG TTA CCT CAG GCT TCC CAT AGC	917
	Arg Ser Trp Ile Gln Met Leu Gln Gln Leu Pro Gln Ala Ser His Ser	
	45 50 55	

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	TTA AAA AAC CTG CTA GAA GAG GAA TGG AAT TTC ACC AAA GAA ATA ACC Leu Lys Asn Leu Leu Glu Glu Glu Trp Asn Phe Thr Lys Glu Ile Thr 60 65 70	965
5	CAT TAT ATC CGT GGC GGA GAA GCG CAG GCT GGA AAG CTT TTC TGT GAC His Tyr Ile Arg Gly Gly Glu Ala Gln Ala Gly Lys Leu Phe Cys Asp 75 80 85	1013
	ATC GCA GGG ATG CTG CTG AAA TCC ACA GGG AGC TTT CTG GAA TCC GGC Ile Ala Gly Met Leu Leu Lys Ser Thr Gly Ser Phe Leu Glu Ser Gly 90 95 100 105	1061
10	CTG CAG GAG AGC TGT GCT GAG CTG TGG ACC AGN GCC GAC GAC AAC GGT Leu Gln Glu Ser Cys Ala Glu Leu Trp Thr Xaa Ala Asp Asp Asn Gly 110 115 120	1109
15	GCT GCC GAC GAG CTA AGG AGA TCT GTC ATC GAG ATC AGC CGA GCA CTC Ala Ala Asp Glu Leu Arg Arg Ser Val Ile Glu Ile Ser Arg Ala Leu 125 130 135	1157
	AAG GAG CTC TTC CAC GAA GCC AGG GAA AGA GCC TCC AAG GCC CTG GGC Lys Glu Leu Phe His Glu Ala Arg Glu Arg Ala Ser Lys Ala Leu Gly 140 145 150	1205
20	TTT GCT AAA ATG CTG AGG AAG GAC CTA GAA ATA GCA GCA GAG TTC GTG Phe Ala Lys Met Leu Arg Lys Asp Leu Glu Ile Ala Ala Glu Phe Val 155 160 165	1253
	CTA TCT GCA TCA GCC CGA GAG CTC CTG GAC GCT CTG AAA GCA AAG CAG Leu Ser Ala Ser Ala Arg Glu Leu Leu Asp Ala Leu Lys Ala Lys Gln 170 175 180 185	1301
25	TAT GTT AAG GTA CAG ATT CCC GGG TTA GAG AAT TTG CAC GTG TTT GTC Tyr Val Lys Val Gln Ile Pro Gly Leu Glu Asn Leu His Val Phe Val 190 195 200	1349
30	CCC GAC AGC CTC GCT GAG GAG AAG AAA ATT ATT TTG CAG CTA CTC AAT Pro Asp Ser Leu Ala Glu Glu Lys Lys Ile Ile Leu Gln Leu Leu Asn 205 210 215	1397
	GCT GCC ACA GGA AAG GAC TGC TCA AAG GAT CCA GAC GAC GTC TTC ATG Ala Ala Thr Gly Lys Asp Cys Ser Lys Asp Pro Asp Asp Val Phe Met 220 225 230	1445
35	GAT GCC TTC CTG CTC CTG ACC AAG CAT GGG GAC CGA GCC CGT GAC TCA Asp Ala Phe Leu Leu Leu Thr Lys His Gly Asp Arg Ala Arg Asp Ser 235 240 245	1493
	GAA GAT GGC TGG GGC ACA TGG GAA GCT CGG GCT GTC AAA ATT GTG CCT Glu Asp Gly Trp Gly Thr Trp Glu Ala Arg Ala Val Lys Ile Val Pro 250 255 260 265	1541
40	CAG GTG GAG ACT GTG GAC ACC CTG AGA AGC ATG CAG GTG GAC AAC CTT Gln Val Glu Thr Val Asp Thr Leu Arg Ser Met Gln Val Asp Asn Leu 270 275 280	1589
45	CTG CTG GTT GTC ATG GAG TCT GCT CAC CTC GTA CTT CAG AGA AAA GCC Leu Leu Val Val Met Glu Ser Ala His Leu Val Leu Gln Arg Lys Ala 285 290 295	1637
	TTC CAG CAG TCC ATT GAG GGG CTG ATG ACT GTA CGC CAT GAG CAG ACA Phe Gln Gln Ser Ile Glu Gly Leu Met Thr Val Arg His Glu Gln Thr 300 305 310	1685
50	TCT AGC CAG CCC ATC ATC GCC AAA GGT TTG CAG CAG CTC AAG AAC GAT Ser Ser Gln Pro Ile Ile Ala Lys Gly Leu Gln Gln Leu Lys Asn Asp 315 320 325	1733
	GCA CTT GAG CTA TGC AAC AGA ATC AGC GAT GCC ATC GAC CGT GTG GAC Ala Leu Glu Leu Cys Asn Arg Ile Ser Asp Ala Ile Asp Arg Val Asp 330 335 340 345	1781

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	CAC ATG TTC ACC CTG GAG TTC GAT GCT GAG GTC GAG GAG TCT GAG TCG His Met Phe Thr Leu Glu Phe Asp Ala Glu Val Glu Glu Ser Glu Ser 350 355 360	1829
5	GCC ACG CTG CAG CAG TAC TAC CGA GAA GCC ATG ATT CAG GGC TAC AAC Ala Thr Leu Glu Gln Tyr Tyr Arg Glu Ala Met Ile Gln Gly Tyr Asn 365 370 375	1877
	TTT GGG TTT GAG TAT CAT AAA GAA GTT GTT CGT TTG ATG TCT GGG GAA Phe Gly Phe Glu Tyr His Lys Glu Val Val Arg Leu Met Ser Gly Glu 380 385 390	1925
10	TTC AGG CAG AAG ATA GGA GAC AAA TAT ATA ACG TTC GCC CAG AAG TGG Phe Arg Gln Lys Ile Gly Asp Lys Tyr Ile Thr Phe Ala Gln Lys Trp 395 400 405	1973
15	ATG AAT TAC GTG CTG ACC AAA TGC GAG AGC GGC AGA GGC ACA AGA CCC Met Asn Tyr Val Leu Thr Lys Cys Glu Ser Gly Arg Gly Thr Arg Pro 410 415 420 425	2021
	AGA TGG GCC ACC CAA GGA TTT GAT TTC CTA CAA GCC ATT GAA CCT GCC Arg Trp Ala Thr Gln Gly Phe Asp Phe Leu Gln Ala Ile Glu Pro Ala 430 435 440	2069
20	TTT ATT TCA GCT TTA CCA GAA GAT GAC TTC TTG AGT TTG CAA GCC CTG Phe Ile Ser Ala Leu Pro Glu Asp Asp Phe Leu Ser Leu Gln Ala Leu 445 450 455	2117
	ATG AAT GAG TGC ATC GGG CAC GTC ATA GGA AAG CCA CAC AGC CCT GTC Met Asn Glu Cys Ile Gly His Val Ile Gly Lys Pro His Ser Pro Val 460 465 470	2165
25	ACA GCT ATC CAT CGG AAC AGC CCC CGC CCT GTG AAG GTG CCC CGA TGC Thr Ala Ile His Arg Asn Ser Pro Arg Pro Val Lys Val Pro Arg Cys 475 480 485	2213
30	CAC AGT GAC CCT CCT AAC CCT CAC CTC ATC ATC CCG ACT CCA GAG GGA His Ser Asp Pro Pro Asn Pro His Leu Ile Ile Pro Thr Pro Glu Gly 490 495 500 505	2261
	TTC AGG GGT TCC AGT GTC CCT GAA AAC GAC CGC TTG GCC TCC ATA GCT Phe Arg Gly Ser Ser Val Pro Glu Asn Asp Arg Leu Ala Ser Ile Ala 510 515 520	2309
35	GCA GAA CTG CAG TTC AGG TCT CTG AGT CGG CAC TCA AGC CCC ACG GAA Ala Glu Leu Phe Arg Ser Leu Ser Arg His Ser Ser Pro Thr Glu 525 530 535	2357
	GAG CGA GAC GAG CCA GCG TAT CCT CGG AGT GAC TCA AGT GGA TCA ACT Glu Arg Asp Glu Pro Ala Tyr Pro Arg Ser Asp Ser Ser Gly Ser Thr 540 545 550	2405
40	CGG AGA AGC TGG GAA CTT CGA ACA CTC ATC AGC CAG ACC AAA GAC TCG Arg Arg Ser Trp Glu Leu Arg Thr Leu Ile Ser Gln Thr Lys Asp Ser 555 560 565	2453
45	GCC TCT AAG CAG GGG CCC ATA GAA GCT ATC CAG AAG TCA GTC CGA CTG Ala Ser Lys Gln Gly Pro Ile Glu Ala Ile Gln Lys Ser Val Arg Leu 570 575 580 585	2501
	TTT GAA GAG AGG AGG TAT CGA GAG ATG AGG AGA AAG AAT ATC ATC GGC Phe Glu Glu Arg Arg Tyr Arg Glu Met Arg Arg Lys Asn Ile Ile Gly 590 595 600	2549
50	CAA GTG TGC GAT ACC CCT AAG TCC TAT GAT AAC GTC ATG CAT GTT GGA Gln Val Cys Asp Thr Pro Lys Ser Tyr Asp Asn Val Met His Val Gly 605 610 615	2597
	CTG AGG AAG GTG ACA TTT AAG TGG CAA AGA GGA AAC AAA ATT GGA GAA Leu Arg Lys Val Thr Phe Lys Trp Gln Arg Gly Asn Lys Ile Gly Glu 620 625 630	2645

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	GGA CAG TAT GGA AAA GTA TAC ACC TGC ATC AGT GTT GAC ACA GGG GAG Gly Gln Tyr Gly Lys Val Tyr Thr Cys Ile Ser Val Asp Thr Gly Glu 635 640 645	2693
5	CTG ATG GCC ATG AAG GAG ATT CGA TTT CAG CCT AAC GAC CAC AAG ACT Leu Met Ala Met Lys Glu Ile Arg Phe Gln Pro Asn Asp His Lys Thr 650 655 660 665	2741
	ATC AAG GAG ACT GCA GAC GAG TTG AAA ATA TTT GAA GGC ATC AAG CAC Ile Lys Glu Thr Ala Asp Glu Leu Lys Ile Phe Glu Gly Ile Lys His 670 675 680	2789
10	CCC AAC CTG GTC CGG TAT TTT GGC GTG GAG CTT CAC AGG GAA GAG ATG Pro Asn Leu Val Arg Tyr Phe Gly Val Glu Leu His Arg Glu Glu Met 685 690 695	2837
15	TAC ATC TTC ATG GAG TAC TGT GAT GAG GGT ACA CTA GAG GAG GTG TCA Tyr Ile Phe Met Glu Tyr Cys Asp Glu Gly Thr Leu Glu Glu Val Ser 700 705 710	2885
	CGA CTG GGC CTG CAG GAG CAC GTC ATC AGG TTA TAT ACC AAG CAG ATC Arg Leu Gly Leu Gln Glu His Val Ile Arg Leu Tyr Thr Lys Gln Ile 715 720 725	2933
20	ACT GTC GCC ATC AAC GTC CTC CAT GAG CAC GGC ATC GTT CAC CGA GAC Thr Val Ala Ile Asn Val Leu His Glu His Gly Ile Val His Arg Asp 730 735 740 745	2981
	ATC AAA GGT GCC AAT ATC TTC CTT ACG TCA TCT GGA CTA ATC AAG CTG Ile Lys Gly Ala Asn Ile Phe Leu Thr Ser Ser Gly Leu Ile Lys Leu 750 755 760	3029
25	GGA GAT TTT GGA TGC TCT GTA AAA CTT AAA AAC AAC GCC CAG ACC ATG Gly Asp Phe Gly Cys Ser Val Lys Leu Lys Asn Asn Ala Gln Thr Met 765 770 775	3077
30	CCC GGA GAG GTG AAC AGC ACC CTA GGG ACA GCA GCT TAC ATG GCC CCT Pro Gly Glu Val Asn Ser Thr Leu Gly Thr Ala Ala Tyr Met Ala Pro 780 785 790	3125
	GAA GTT ATT ACC CGA GCC AAA GGA GAA GGC CAC GGA CGT GCG GCA GAT Glu Val Ile Thr Arg Ala Lys Gly Glu Gly His Gly Arg Ala Ala Asp 795 800 805	3173
35	ATC TGG AGT CTG GGG TGC GTC GTC ATA GAG ATG GTG ACT GGC AAG CGG Ile Trp Ser Leu Gly Cys Val Val Ile Glu Met Val Thr Gly Lys Arg 810 815 820 825	3221
	CCT TGG CAT GAG TAT GAA CAC AAC TTT CAG ATT ATG TAC AAG GTG GGG Pro Trp His Glu Tyr Glu His Asn Phe Gln Ile Met Tyr Lys Val Gly 830 835 840	3269
40	ATG GGA CAC AAG CCA CCA ATC CCG GAA AGG CTA AGC CCT GAA GGA AAG Met Gly His Lys Pro Pro Ile Pro Glu Arg Leu Ser Pro Glu Gly Lys 845 850 855	3317
45	GCC TTT CTC TCG CAC TGC CTG GAA AGT GAC CCG AAG ATA CGG TGG ACA Ala Phe Leu Ser His Cys Leu Glu Ser Asp Pro Lys Ile Arg Trp Thr 860 865 870	3365
	GCC AGC CAG CTC CTC GAC CAC GCT TTT GTC AAG GTT TGC ACA GAT GAA Ala Ser Gln Leu Leu Asp His Ala Phe Val Lys Val Cys Thr Asp Glu 875 880 885	3413
50	GAG T GAAGTGAACC AGTCCGTGGC CTAGTAGTGT GTGCACAGAA TCCCGTGATC Glu 890	3467
55	ACTACTGTAT GTAATATTTA CATAAGACT GCAGCGCAGG CGGCCTTCCT AACCTCCAG GACTGAAGAC TACAGGGGTG ACAAGCCTCA CTTCTGCTGC TCCTGTCCGC TGCTGAGTGA CAGTGCTGAG GTTAAAGGAG CCGCACGTTA AGTGCCATTA CTACTGTACA CGGCCACCGC CTCTGTCCCC TCCGACCCTC TCGTGACTGA GAACCAACCG TGTCATCAGC ACAGTGTITT TGAGCTCCTG GGGTTCAGAA GAACATGTAG TGTTCCCGGG TGTCGGGAC GTTTATTTCA ACCTCCTGGT CGTTGGCTCT GACTGTGGAG CCTCCTTGT CGAAAGCTGC AGGTTTGTTA	3527 3587 3647 3707 3767 3827

TCGAAAGGCT CGTAAGTGAA GCTGAAGAAA AGGTTCTTTT TCAATAAATG GTTTATTTTA 3887
GGAAAGCGAA AAAAAAAAAA AAAAAA 3913

Table 5.

10	AAGAAGAAGG ACAGGGAGCA GAGGGGACAA GAAAACACGG CTGCTTTCTG GTTCAACCGA TCGAACGAAC TGATCTGGTT AGAACTGCAG GCCTGGCAGC CGGGCCGCAC CATCAATGAC CAGGACCTCT TTCTCTACAC AGCCCCCAGC GCCATCCCAG ACATCATCAA TGAGATCCTC ACCTTCAAAG TTAACACAGG GAGCAATTGCC TTCTCCAGCA ATGGAGCCGG TTTC AACGGG CCCTTGGTAG AAGGCCAGTG CAGAACCCCT CAGGAGACAA ACCGTGTGGG CTGCTCATCG	60 120 180 240 300
15	TACCACGAGC ACCTCCAGCG CCAGAGGGTC TCGTTTGAGC AGGTGAAGCG GATA ATG Met 1	357
	GAG CTG CTG GAG TAC ATG GAG GCA CTT TAC CCA TCC TTG CAG GCT CTG Glu Leu Leu Glu Tyr Met Glu Ala Leu Tyr Pro Ser Leu Gln Ala Leu 5 10 15	405
20	CAG AAG GAC TAT GAA CGG TAC GCC AAG GAC TTT GAG GAC AGA GTG Gln Lys Asp Tyr Glu Arg Tyr Ala Lys Asp Phe Glu Asp Arg Val 20 25 30	453
25	CAG GCG CTC TGC CTG TGG CTC AAC ATC ACG AAA GAT CTA AAT CAG AAG Gln Ala Leu Cys Leu Trp Leu Asn Ile Thr Lys Asp Leu Asn Gln Lys 35 40 45	501
	CTG CGG ATC ATG GGC ACC GTG CTG GGC ATC AAG TTC CTA TCA GAC ATT Leu Arg Ile Met Gly Thr Val Leu Gly Ile Lys Phe Leu Ser Asp Ile 50 55 60 65	549
30	GGC TGG CCA GTG AAA GAA ATC CCC TCC CCT CGG CCG TCC AAG GGC TAC Gly Trp Pro Val Lys Glu Ile Pro Ser Pro Arg Pro Ser Lys Gly Tyr 70 75 80	597
	GAG CCA GAG GAC GAG GTC GAG GAC ACG GAG GTT GAG CTG AGG GAG CTG Glu Pro Glu Asp Glu Val Glu Asp Thr Glu Val Glu Leu Arg Glu Leu 85 90 95	645
35	GAG AGC GGG ACG GAG GAG AGT GAC GAG GAG CCA ACC CCC AGT CCG AGG Glu Ser Gly Thr Glu Glu Ser Asp Glu Glu Pro Thr Pro Ser Pro Arg 100 105 110	693
40	GTG CCA GAG CTC AGG CTG TCC ACA GAC ACC ATC TTG GAC AGT CCG TCC Val Pro Glu Leu Arg Leu Ser Thr Asp Thr Ile Leu Asp Ser Arg Ser 115 120 125	741
	CAG GGC TGC GTC TCC AGG AAG CTG GAG AGG CTC GAG TCA GAG GAA GAT Gln Gly Cys Val Ser Arg Lys Leu Glu Arg Leu Glu Ser Glu Glu Asp 130 135 140 145	789
45	TCC ATA GGC TGG GGG ACA GCG GAC TGT GGC CCT GAA GCC AGC AGG CAT Ser Ile Gly Trp Gly Thr Ala Asp Cys Gly Pro Glu Ala Ser Arg His 150 155 160	837
	TGT TTG ACT TCT ATC TAT AGA CCA TTC GTG GAC AAA GCA CTG AAG CAA Cys Leu Thr Ser Ile Tyr Arg Pro Phe Val Asp Lys Ala Leu Lys Gln 165 170 175	885

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	ATG GGG CTA AGA AAG TTA ATT TTA CGA CTT CAT AAG CTT ATG AAT GGG Met Gly Leu Arg Lys Leu Ile Leu Arg Leu His Lys Leu Met Asn Gly 180 185 190	933
5	TCC TTG CAA AGA GCT CGT GTA GCT CTG GTG AAG GAC GAC CGT CCA GTG Ser Leu Gln Arg Ala Arg Val Ala Leu Val Lys Asp Asp Arg Pro Val 195 200 205	981
	GAG TTC TCT GAC TTT CCA GGT CCC ATG TGG GGC TCG GAT TAT GTG CAG Glu Phe Ser Asp Phe Pro Gly Pro Met Trp Gly Ser Asp Tyr Val Gln 210 215 220 225	1029
10	TTG TCG GGA ACA CCT CCT TCC TCA GAG CAG AAG TGT AGC GCT GTG TCC Leu Ser Gly Thr Pro Pro Ser Ser Glu Gln Lys Cys Ser Ala Val Ser 230 235 240	1077
15	TGG GAA GAA CTG AGA GCC ATG GAC CTG CCT TCC TTT GAG CCC GCC TTC Trp Glu Glu Leu Arg Ala Met Asp Leu Pro Ser Phe Glu Pro Ala Phe 245 250 255	1125
	CTG GTG CTC TGT CGG GTC CTG CTG AAC GTG ATC CAC GAG TGC CTG AAG Leu Val Leu Cys Arg Val Leu Leu Asn Val Ile His Glu Cys Leu Lys 260 265 270	1173
20	CTG CGG CTG GAA CAG AGG CCT GCC GGG GAG CCT TCC CTC TTG AGT ATC Leu Arg Leu Glu Gln Arg Pro Ala Gly Glu Pro Ser Leu Leu Ser Ile 275 280 285	1221
	AAA CAG CTA GTG CGA GAG TGT AAA GAG GTC CTA AAG GGC GGG CTC CTG Lys Gln Leu Val Arg Glu Cys Lys Glu Val Leu Lys Gly Gly Leu Leu 290 295 300 305	1269
25	ATG AAG CAG TAT TAC CAG TTC ATG CTG CAG GAG GTC CTG GGC GGA CTG Met Lys Gln Tyr Tyr Gln Phe Met Leu Gln Glu Val Leu Gly Gly Leu 310 315 320	1317
30	GAG AAG ACC GAC TGC AAC ATG GAT GCC TTT GAG GAG GAC CTG CAG AAG Glu Lys Thr Asp Cys Asn Met Asp Ala Phe Glu Glu Asp Leu Gln Lys 325 330 335	1365
	ATG CTG ATG GTG TAT TTT GAT TAC ATG AGA AGC TGG ATC CAA ATG CTA Met Leu Met Val Tyr Phe Asp Tyr Met Arg Ser Trp Ile Gln Met Leu 340 345 350	1413
35	CAG CAG TTA CCT CAG GCT TCC CAT AGC TTA AAA AAC CTG CTA GAA GAG Gln Gln Leu Pro Gln Ala Ser His Ser Leu Lys Asn Leu Leu Glu Glu 355 360 365	1461
	GAA TGG AAT TTC ACC AAA GAA ATA ACC CAT TAT ATC CGT GGC GGA GAA Glu Trp Asn Phe Thr Lys Glu Ile Thr His Tyr Ile Arg Gly Gly Glu 370 375 380 385	1509
40	GCG CAG GCT GGA AAG CTT TTC TGT GAC ATC GCA GGG ATG CTG CTG AAA Ala Gln Ala Gly Lys Leu Phe Cys Asp Ile Ala Gly Met Leu Leu Lys 390 395 400	1557
45	TCC ACA GGG AGC TTT CTG GAA TCC GGC CTG CAG GAG AGC TGT GCT GAG Ser Thr Gly Ser Phe Leu Glu Ser Gly Leu Gln Glu Ser Cys Ala Glu 405 410 415	1605
	CTG TGG ACC AGC GCC GAC GAC AAC GGT GCT GCC GAC GAG CTA AGG AGA Leu Trp Thr Ser Ala Asp Asp Asn Gly Ala Ala Asp Glu Leu Arg Arg 420 425 430	1653
50	TCT GTC ATC GAG ATC AGC CGA GCA CTC AAG GAG CTC TTC CAC GAA GCC Ser Val Ile Glu Ile Ser Arg Ala Leu Lys Glu Leu Phe His Glu Ala 435 440 445	1701
	AGG GAA AGA GCC TCC AAG GCC CTG GGC TTT GCT AAA ATG CTG AGG AAG Arg Glu Arg Ala Ser Lys Ala Leu Gly Phe Ala Lys Met Leu Arg Lys 450 455 460 465	1749

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	GAC CTA GAA ATA GCA GCA GAG TTC GTG CTA TCT GCA TCA GCC CGA GAG Asp Leu Glu Ile Ala Ala Glu Phe Val Leu Ser Ala Ser Ala Arg Glu 470 475 480	1797
5	CTC CTG GAC GCT CTG AAA GCA AAG CAG TAT GTT AAG GTA CAG ATT CCC Leu Leu Asp Ala Leu Lys Ala Lys Gln Tyr Val Lys Val Gln Ile Pro 485 490 495	1845
	GGG TTA GAG AAT TTG CAC GTG TTT GTC CCC GAC AGC CTC GCT GAG GAG Gly Leu Glu Asn Leu His Val Phe Val Pro Asp Ser Leu Ala Glu Glu 500 505 510	1893
10	AAG AAA ATT ATT TTG CAG CTA CTC AAT GCT GCC ACA GGA AAG GAC TGC Lys Lys Ile Ile Leu Gln Leu Leu Asn Ala Ala Thr Gly Lys Asp Cys 515 520 525	1941
15	TCA AAG GAT CCA GAC GAC GTC TTC ATG GAT GCC TTC CTG CTC CTG ACC Ser Lys Asp Pro Asp Asp Val Phe Met Asp Ala Phe Leu Leu Leu Thr 530 535 540 545	1989
	AAG CAT GGG GAC CGA GCC CGT GAC TCA GAA GAT GGC TGG GGC ACA TGG Lys His Gly Asp Arg Ala Arg Asp Ser Glu Asp Gly Trp Gly Thr Trp 550 555 560	2037
20	GAA GCT CGG GCT GTC AAA ATT GTG CCT CAG GTG GAG ACT GTG GAC ACC Glu Ala Arg Ala Val Lys Ile Val Pro Gln Val Glu Thr Val Asp Thr 565 570 575	2085
	CTG AGA AGC ATG CAG GTG GAC AAC CTT CTG CTG GTT GTC ATG GAG TCT Leu Arg Ser Met Gln Val Asp Asn Leu Leu Leu Val Val Met Glu Ser 580 585 590	2133
25	GCT CAC CTC GTA CTT CAG AGA AAA GCC TTC CAG CAG TCC ATT GAG GGG Ala His Leu Val Leu Gln Arg Lys Ala Phe Gln Gln Ser Ile Glu Gly 595 600 605	2181
30	CTG ATG ACT GTA CGC CAT GAG CAG ACA TCT AGC CAG CCC ATC ATC GCC Leu Met Thr Val Arg His Glu Gln Thr Ser Ser Gln Pro Ile Ile Ala 610 615 620 625	2229
	AAA GGT TTG CAG CAG CTC AAG AAC GAT GCA CTT GAG CTA TGC AAC AGA Lys Gly Leu Gln Gln Leu Lys Asn Asp Ala Leu Glu Leu Cys Asn Arg 630 635 640	2277
35	ATC AGC GAT GCC ATC GAC CGT GTG GAC CAC ATG TTC ACC CTG GAG TTC Ile Ser Asp Ala Ile Asp Arg Val Asp His Met Phe Thr Leu Glu Phe 645 650 655	2325
	GAT GCT GAG GTC GAG GAG TCT GAG TCG GCC ACG CTG CAG CAG TAC TAC Asp Ala Glu Val Glu Glu Ser Glu Ser Ala Thr Leu Gln Gln Tyr Tyr 660 665 670	2373
40	CGA GAA GCC ATG ATT CAG GGC TAC AAC TTT GGG TTT GAG TAT CAT AAA Arg Glu Ala Met Ile Gln Gly Tyr Asn Phe Gly Phe Glu Tyr His Lys 675 680 685	2421
45	GAA GTT GTT CGT TTG ATG TCT GGG GAA TTC AGG CAG AAG ATA GGA GAC Glu Val Val Arg Leu Met Ser Gly Glu Phe Arg Gln Lys Ile Gly Asp 690 695 700 705	2469
	AAA TAT ATA AGC TTC GCC CAG AAG TGG ATG AAT TAC GTG CTG ACC AAA Lys Tyr Ile Ser Phe Ala Gln Lys Trp Met Asn Tyr Val Leu Thr Lys 710 715 720	2517
50	TGC GAG AGC GGC AGA GGC ACA AGA CCC AGA TGG GCC ACC CAA GGA TTT Cys Glu Ser Gly Arg Gly Thr Arg Pro Arg Trp Ala Thr Gln Gly Phe 725 730 735	2565
	GAT TTC CTA CAA GCC ATT GAA CCT GCC TTT ATT TCA GCT TTA CCA GAA Asp Phe Leu Gln Ala Ile Glu Pro Ala Phe Ile Ser Ala Leu Pro Glu 740 745 750	2613

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	GAT GAC TTC TTG AGT TTG CAA GCC CTG ATG AAT GAG TGC ATC GGG CAC Asp Asp Phe Leu Ser Leu Gln Ala Leu Met Asn Glu Cys Ile Gly His 755 760 765	2661
5	GTC ATA GGA AAG CCA CAC AGC CCT GTC ACA GCT ATC CAT CGG AAC AGC Val Ile Gly Lys Pro His Ser Pro Val Thr Ala Ile His Arg Asn Ser 770 775 780 785	2709
	CCC CGC CCT GTG AAG GTG CCC CGA TGC CAC AGT GAC CCT CCT AAC CCT Pro Arg Pro Val Lys Val Pro Arg Cys His Ser Asp Pro Pro Asn Pro 790 795 800	2757
10	CAC CTC ATC ATC CCG ACT CCA GAG GGA TTC AGC ACC CGG AGC GTG CCT His Leu Ile Ile Pro Thr Pro Glu Gly Phe Ser Thr Arg Ser Val Pro 805 810 815	2805
15	TCC GAC GCT CGG ACC CAT GGC AAC TCT GTT GCT GCT GCT GCT GTT Ser Asp Ala Arg Thr His Gly Asn Ser Val Ala Ala Ala Ala Val 820 825 830	2853
	CGT GCC GCC GCC ACC ACT GCT GCT GGC CGC CCT GGC CCA GGT GGT GGT Arg Ala Ala Ala Thr Thr Ala Ala Gly Arg Pro Gly Pro Gly Gly Gly 835 840 845	2901
20	GAC TCT GTG CCA GCC AAA CCT GTC AAC ACT GCC CCT GAT ACC AGG GGT Asp Ser Val Pro Ala Lys Pro Val Asn Thr Ala Pro Asp Thr Arg Gly 850 855 860 865	2949
	TCC AGT GTC CCT GAA AAC GAC CGC TTG GCC TCC ATA GCT GCA GAA CTG Ser Ser Val Pro Glu Asn Asp Arg Leu Ala Ser Ile Ala Ala Glu Leu 870 875 880	2997
25	CAG TTC AGG TCT CTG AGT CGG CAC TCA AGC CCC ACG GAA GAG CGA GAC Gln Phe Arg Ser Leu Ser Arg His Ser Ser Pro Thr Glu Glu Arg Asp 885 890 895	3045
30	GAG CCA GCG TAT CCT CGG AGT GAC TCA AGT GGA TCA ACT CGG AGA AGC Glu Pro Ala Tyr Pro Arg Ser Asp Ser Ser Gly Ser Thr Arg Arg Ser 900 905 910	3093
	TGG GAA CTT CGA ACA CTC ATC AGC CAG ACC AAA GAC TCG GCC TCT AAG Trp Glu Leu Arg Thr Leu Ile Ser Gln Thr Lys Asp Ser Ala Ser Lys 915 920 925	3141
35	CAG GGG CCC ATA GAA GCT ATC CAG AAG TCA GTC CGA CTG TTT GAA GAG Gln Gly Pro Ile Glu Ala Ile Gln Lys Ser Val Arg Leu Phe Glu Glu 930 935 940 945	3189
	AGG AGG TAT CGA GAG ATG AGG AGA AAG AAT ATC ATC GGC CAA GTG TGC Arg Arg Tyr Arg Glu Met Arg Arg Lys Asn Ile Ile Gly Gln Val Cys 950 955 960	3237
40	GAT ACC CCT AAG TCC TAT GAT AAC GTC ATG CAT GTT GGA CTG AGG AAG Asp Thr Pro Lys Ser Tyr Asp Asn Val Met His Val Gly Leu Arg Lys 965 970 975	3285
45	GTG ACA TTT AAG TGG CAA AGA GGA AAC AAA ATT GGA GAA GGA CAG TAT Val Thr Phe Lys Trp Gln Arg Gly Asn Lys Ile Gly Glu Gly Gln Tyr 980 985 990	3333
	GGA AAA GTA TAC ACC TGC ATC AGT GTT GAC ACA GGG GAG CTG ATG GCC Gly Lys Val Tyr Thr Cys Ile Ser Val Asp Thr Gly Glu Leu Met Ala 995 1000 1005	3381
50	ATG AAG GAG ATT CGA TTT CAG CCT AAC GAC CAC AAG ACT ATC AAG GAG Met Lys Glu Ile Arg Phe Gln Pro Asn Asp His Lys Thr Ile Lys Glu 1010 1015 1020 1025	3429
	ACT GCA GAC GAG TTG AAA ATA TTT GAA GGC ATC AAG CAC CCC AAC CTG Thr Ala Asp Glu Leu Lys Ile Phe Glu Gly Ile Lys His Pro Asn Leu 1030 1035 1040	3477

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	GTC CGG TAT TTT GGC GTG GAG CTT CAC AGG GAA GAG ATG TAC ATC TTC Val Arg Tyr Phe Gly Val Glu Leu His Arg Glu Glu Met Tyr Ile Phe 1045 1050 1055	3525
5	ATG GAG TAC TGT GAT GAG GGT ACA CTA GAG GAG GTG TCA CGA CTG GGC Met Glu Tyr Cys Asp Glu Gly Thr Leu Glu Glu Val Ser Arg Leu Gly 1060 1065 1070	3573
	CTG CAG GAG CAC GTC ATC AGG TTA TAT ACC AAG CAG ATC ACT GTC GCC Leu Gln Glu His Val Ile Arg Leu Tyr Thr Lys Gln Ile Thr Val Ala 1075 1080 1085	3621
10	ATC AAC GTC CTC CAT GAG CAC GGC ATC GTT CAC CGA GAC ATC AAA GGT Ile Asn Val Leu His Glu His Gly Ile Val His Arg Asp Ile Lys Gly 1090 1095 1100 1105	3669
15	GCC AAT ATC TTC CTT ACG TCA TCT GGA CTA ATC AAG CTG GGA GAT TTT Ala Asn Ile Phe Leu Thr Ser Ser Gly Leu Ile Lys Leu Gly Asp Phe 1110 1115 1120	3717
	GGA TGC TCT GTA AAA CTT AAA AAC AAC GCC CAG ACC ATG CCC GGA GAG Gly Cys Ser Val Lys Leu Lys Asn Asn Ala Gln Thr Met Pro Gly Glu 1125 1130 1135	3765
20	GTG AAC AGC ACC CTA GGG ACA GCA GCT TAC ATG GCC CCT GAA GTT ATT Val Asn Ser Thr Leu Gly Thr Ala Ala Tyr Met Ala Pro Glu Val Ile 1140 1145 1150	3813
	ACC CGA GCC AAA GGA GAA GGC CAC GGA CGT GCG GCA GAT ATC TGG AGT Thr Arg Ala Lys Gly Glu Gly His Gly Arg Ala Ala Asp Ile Trp Ser 1155 1160 1165	3861
25	CTG GGG TGC GTC GTC ATA GAG ATG GTG ACT GGC AAG CGG CCT TGG CAT Leu Gly Cys Val Val Ile Glu Met Val Thr Gly Lys Arg Pro Trp His 1170 1175 1180 1185	3909
30	GAG TAT GAA CAC AAC TTT CAG ATT ATG TAC AAG GTG GGG ATG GGA CAC Glu Tyr Glu His Asn Phe Gln Ile Met Tyr Lys Val Gly Met Gly His 1190 1195 1200	3957
	AAG CCA CCA ATC CCG GAA AGG CTA AGC CCT GAA GGA AAG GCC TTT CTC Lys Pro Pro Ile Pro Glu Arg Leu Ser Pro Glu Gly Lys Ala Phe Leu 1205 1210 1215	4005
35	TCG CAC TGC CTG GAA AGT GAC CCG AAG ATA CCG TGG ACA GCC AGC CAG Ser His Cys Leu Glu Ser Asp Pro Lys Ile Arg Trp Thr Ala Ser Gln 1220 1225 1230	4053
	CTC CTC GAC CAC GCT TTT GTC AAG GTT TGC ACA GAT GAA GAG Leu Leu Asp His Ala Phe Val Lys Val Cys Thr Asp Glu Glu 1235 1240 1245	4095
40	TGAAGTGAAC CAGTCCGTGG CTTAGTAGTG TGTGGACAGA ATCCCGTGAT CACTACTETA TGTAATATTT ACATAAGAC TGCAGCGCAG GCGGCCTTCC TAACCTCCCA GGAAGTGAAGA CTACAGGGGT GACAAGCCTC ACTTCTGCTG CTCCTGTCGC CTGCTGAGTG ACAGTGCTGA GGTTAAAGGA GCCGCACGTT AAGTGCCATT ACTACTGTAC ACGGCCACCG CCTCTGTCCC CTCCGACCCCT CTCGTGACTG AGAACCACCC GTGTATCAG CACAGTGTTT TTGAGTCCT GGGGTTTCTGAGA AGAACATGTA GTGTTCCCGG GTGTCCGGGA CGTTTATTTT AACCTCTGG TCGTTGGCTC TGACTGTGGA GCCTCCTTGT TCGAAAGCTG CAGGTTTGT ATGCAAAGGC TCGTAAGTGA AGCTGAAGAA AAGGTTCTTT TTCAATAAAT GGTATTATTT AGGAAAGCGA AAAAAAAAA AAAAAAA	4155 4215 4275 4335 4395 4455 4515 4575 4592

MEKK 5 represents a splice variant of MEKK 4. The splice
insert is shown by the underlined portion of the sequence
shown in Table 5.

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The amino acid sequences for MEKK 2 and MEKK 3 compared with the amino acid sequence of MEKK 1 are shown in Table 6.

Table 6.

5	MVTAVPAVFSKLVTHLNASGSTHFTDRRLMALADEVEIAEVIQLGVEDTVGQDQSL MEKK 1	1
	NDGQALNSINQDI-----	2
	NDGEALDSINQDLVALQNSRTRL-	3
	AVAPTSCLNSSLNITVIREKTKGLSATRLSASSEDIIDRLAGVSVGLPSSTTTEQPKP	1
10	AVLMDPVGHLYLYOCPGKONLHKKQKRMFESNLNTEEEKRILQVTPVKLEDLRSCSKI	2
	S--GYETMKQKDTGHPKRSQDVRICKFENNGERRI-IAFSRPVRYEDVEHKVTTVFQGPLD	3
	AVGTGGRPHSQCLNSS-PLSHAQINFPAPSAPCSSAPSVPIKSRPQAFVPCKIPASAP	1
	AFGQSDNLHYTHNELVPLTTQDQDKAVELLDRSINMKSL-KILLVWNGSTQA-TNLEP	2
	LHYTHNELSILLKQDQDKATDILDRSSSNKSLRILLSSQDRNHTSSSPHSGVSRQVRI	3
15	QTQKFSLOQRNCSHRDQQLSPVFTQS-RPPSSNTHRPKPSRPVPGSTSKLQDATK	1
	SPSPEDLNHTPLGAERKQQLSVGPPMR--DRSSPPPGYIPDILHQIARNGSFTSINSEG	2
	KPSQAGDINTIYQAEPRSRRLSVSSQMPGRSSPPPGYVPERQHIARQGSYTSINSEG	3
	SSNTLDLGSASRCDDSFSGGGNSGNVIPSDETFTVFTVEDKCRLDVNTLNSSI EDLLEA	1
	EFIPESMDQ-MLDPLSLSSPENSGSGCPSLDSPLDGESYPKSRMPRAQSYPDNHQEFDT	2
	EFIPETSEQCMLDPLSSAENSLSGSCASLDRSADSPSFRKSQMSRARSFPQNR---ECSO	3
20	SNPSSDOTTVTFKSEVAVLSPEKAENDTYKDDVNHQKCKEKEAEAEAEALATAMANSAS	1
	YDNPIFEKFGKGGTYPRRYHVSYHHQYNDGRKTFPRARRTQGTFSRSPVSFSPDHSLS	2
	K----RETQLYDKGVKGGTYPRRYHVSVHHQYNDGRRTFPRIRRHQGNLFTLVPSRSL	3
25	QDALPIVPQLQVENGEDIITIQDTPETLPGHTKAKQPYREDAEMKCGQIGLGAFFSCY	1
	TSSGSSVFTPEYDDSRIRRRGSDIDNPTLTVTDISPPSRSPRAPTHRLGKLLGGGAFCR	2
	STNGENMGVAVQYLDPRGRLRSADSENALTQERNVPTKSPAPINMRREKLLGGGAFCR	3
	QAGDVTETLMAVKQVTVVNTSSEGEVVEALREEIRNQHNLNHPNIIRMLGATCEKSN	1
	VYLCTVDVTGRELAVKQVQFNPESPETSKEVMALECEIQLLQILLHERIVQYTGCLRDPO	2
	VYLCTVDVTGRELASKQVQFDPSPETSKESVALECEIQLLQILQHERIVQYTGCLRDRA	3
30	YMLFTFEMAGGSVAHLLSKYGAFKESVWINTYEQLRLGLSTLREH--Q-IIRQVKGANI	1
	EKTLSTFIELSPGGSIKDQLKAYGALTENVTRKYTRQILEGVHYLHSMIVHROIKGANI	2
	EKILTIEMYNPGGSVQDQLKAYGALTESYTRKYTRQILEGNSYLHSMIVHROIKGANI	3
	LIDSTGQ-RLRIADFQAARLASK-GTGAGEFGQQLGTIAFMAPEVLRGQYGRSCDVM	1
	LRDSTGNIKLGDGFGASKRLQITCLSGTGKSVTG-PY----LMSPEVISCEGYGRKADIV	2
	LRDSAGNVKLGDFGASKRLQITCMSTGIRSVTGTPI----LMSPEVISCEGYGRKADVM	3
35	SVGCATTEMACAPPMNAEKHSNHLALIFKIASATTAPSTPSHLSPLGRDVAVRCLFLOP	1
	SVACTVVENLTKPPM-AEFEA-MAA-IFKIATOPTNPCLPPHVSQYTRQFLKRI FVEAK	2
	SLGCTVVENLTKPPM-AEYEA-MAA-IFKIATOPTNPCLPSHSERGRFLRRIFVEAR	3
	QDRPPSRE-LLKHPVFRITV	1
40	L-RP-SAEELLRHMFVYTH	2
	Q-RP-SAEELLTHHFAQLVY	3

Bold Amino Terminus- Regulatory Domain
Underline sequence- Regulatory hinge Sequence
Bold Italics- Catalytic Domain

Table 7 shows the amino acid sequence of the kinase domain of MEKK 4 compared with the kinase domains of MEKK 1, MEKK 2 and MEKK 3.

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Table 7.

5	..IRFQPNHKTIKETADEELKIFEGIKHPNLVRYFGV..ELHREEM.YI	MEKK4
	TYVRNTSSEQEEVVEALREEIRMMGHLNHPN1IRMLGATCEKSNYNLFIE	MEKK1
	QVGFNPESPETSKEVNALECEIQLLKNLLHERIVQYYGCLRDPQEXTLSI	MEKK2
	QVGFDPDSPETSKEVSALECEIQLLKNLQHERIVQYYGCLRDRAEKILT	MEKK3
10	IFMEYCDGTLLEEVSRGLQEHV.I.RLYTKQITVAINVLHEHGNV	MEKK4
	.MMAGGSVAHLLSKYGAFKESV.V.IN..YTEQLLRGLSYLHENQII	MEKK1
	.FMELSPGGSIKDQLKAYGALTENVTRKYTRQILEGVHYLHSMIV	MEKK2
	.FMEYMPGGSVKDQLKAYGALTESVTRKYTRQILEGMSYLSNMIV	MEKK3

10 The foregoing SEQ ID NO's represent sequences deduced according to methods disclosed in the Examples. It should be noted that since nucleic acid and amino acid sequencing technology is not entirely error-free, the foregoing SEQ ID NO's, at best, represent apparent nucleic acid and amino acid sequences of MEKK proteins of the present invention.

 According to the present invention, an MEKK protein of the present invention can include MEKK proteins that have undergone post-translational modification. Such modification can include, for example, glycosylation (e.g., including addition of N-linked and/or O-linked oligosaccharides) or post-translational conformational changes or post-translational deletions.

 Another embodiment of the present invention is an isolated nucleic acid molecule capable of hybridizing, under stringent conditions, with an MEKK protein gene encoding an MEKK protein of the present invention. In accordance with the present invention, an isolated nucleic acid molecule is a nucleic acid molecule that has been removed from its natural milieu (i.e., that has been subject to human manipulation). As such, "isolated" does not reflect the extent to which the nucleic acid molecule

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has been purified. An isolated nucleic acid molecule can include DNA, RNA, or derivatives of either DNA or RNA.

An isolated nucleic acid molecule of the present invention can be obtained from its natural source either as
5 an entire (i.e., complete) gene or a portion thereof capable of forming a stable hybrid with that gene. As used herein, the phrase "at least a portion of" an entity refers to an amount of the entity that is at least sufficient to have the functional aspects of that entity. For example,
10 at least a portion of a nucleic acid sequence, as used herein, is an amount of a nucleic acid sequence capable of forming a stable hybrid with a particular desired gene (e.g., MEKK genes) under stringent hybridization conditions. An isolated nucleic acid molecule of the
15 present invention can also be produced using recombinant DNA technology (e.g., polymerase chain reaction (PCR) amplification, cloning) or chemical synthesis. Isolated MEKK protein nucleic acid molecules include natural nucleic acid molecules and homologues thereof, including, but not
20 limited to, natural allelic variants and modified nucleic acid molecules in which nucleotides have been inserted, deleted, substituted, and/or inverted in such a manner that such modifications do not substantially interfere with the nucleic acid molecule's ability to encode an MEKK protein
25 of the present invention or to form stable hybrids under stringent conditions with natural nucleic acid molecule isolates of MEKK.

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Preferred modifications to an MEKK protein nucleic acid molecule of the present invention include truncating a full-length MEKK protein nucleic acid molecule by, for example: deleting at least a portion of an MEKK protein nucleic acid molecule encoding a regulatory domain (examples illustrated in Table 6) to produce a constitutively active MEKK protein; deleting at least a portion of an MEKK protein nucleic acid molecule encoding a catalytic domain (examples illustrated in Table 6) to produce an inactive MEKK protein; and modifying the MEKK protein to achieve desired inactivation and/or stimulation of the protein, for example, substituting a codon encoding a lysine residue in the catalytic domain (i.e., phosphotransferase domain) with a methionine residue to inactivate the catalytic domain.

A preferred truncated MEKK nucleic acid molecule encodes a form of an MEKK protein containing a catalytic domain but that lacks a regulatory domain. Preferred catalytic domain truncated MEKK nucleic acid molecules encode residues from about 352 to about 672 of MEKK 1, from about 352 to about 619 of MEKK 2, from about 358 to about 626 of MEKK 3, from about 811 to about 1195 of MEKK 4 or from about 863 to about 1247 of MEKK 5.

Another preferred truncated MEKK nucleic acid molecule encodes a form of an MEKK protein comprising an NH₂-terminal regulatory domain a catalytic domain but lacking a catalytic domain. Preferred regulatory domain truncated MEKK nucleic acid molecules encode residues from about 1 to

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about 369 for MEKK 1, from about 1 to about 335 for MEKK 2, from about 1 to about 360 for MEKK 3, from about 1 to about 825 for MEKK 4 and from about 1 to about 875 for MEKK 5, thereby removing the regulatory domain to form the truncated MEKK molecule.

An isolated nucleic acid molecule of the present invention can include a nucleic acid sequence that encodes at least one MEKK protein of the present invention, examples of such proteins being disclosed herein. Although the phrase "nucleic acid molecule" primarily refers to the physical nucleic acid molecule and the phrase "nucleic acid sequence" primarily refers to the sequence of nucleotides that comprise the nucleic acid molecule, the two phrases can be used interchangeably. As heretofore disclosed, MEKK proteins of the present invention include, but are not limited to, proteins having full-length MEKK protein coding regions, portions thereof, and other MEKK protein homologues.

As used herein, an MEKK protein gene includes all nucleic acid sequences related to a natural MEKK protein gene such as regulatory regions that control production of an MEKK protein encoded by that gene (including, but not limited to, transcription, translation or post-translation control regions) as well as the coding region itself. A nucleic acid molecule of the present invention can be an isolated natural MEKK protein nucleic acid molecule or a homologue thereof. A nucleic acid molecule of the present invention can include one or more regulatory regions, full-

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length or partial coding regions, or combinations thereof. The minimal size of an MEKK protein nucleic acid molecule of the present invention is the minimal size capable of forming a stable hybrid under stringent hybridization conditions with a corresponding natural gene.

An MEKK protein nucleic acid molecule homologue can be produced using a number of methods known to those skilled in the art (see, e.g., Sambrook et al., *ibid.*). For example, nucleic acid molecules can be modified using a variety of techniques including, but not limited to, classic mutagenesis techniques and recombinant DNA techniques, such as site-directed mutagenesis, chemical treatment of a nucleic acid molecule to induce mutations, restriction enzyme cleavage of a nucleic acid fragment, ligation of nucleic acid fragments, polymerase chain reaction (PCR) amplification and/or mutagenesis of selected regions of a nucleic acid sequence, synthesis of oligonucleotide mixtures and ligation of mixture groups to "build" a mixture of nucleic acid molecules and combinations thereof. Nucleic acid molecule homologues can be selected from a mixture of modified nucleic acids by screening for the function of the protein encoded by the nucleic acid (e.g., the ability of a homologue to phosphorylate MEK protein or JEK protein) and/or by hybridization with isolated MEKK protein nucleic acids under stringent conditions.

One embodiment of the present invention is an MEKK protein nucleic acid molecule capable of encoding at least

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a portion of an MEKK protein, or a homologue thereof, as described herein. A preferred nucleic acid molecule of the present invention includes, but is not limited to, a nucleic acid molecule that encodes a protein having at
5 least a portion of an amino acid sequence represented by SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:10, or homologues thereof.

A preferred nucleic acid molecule of the present invention is capable of hybridizing under stringent
10 conditions to a nucleic acid that encodes at least a portion of an MEKK protein, or a homologue thereof. Also preferred is an MEKK protein nucleic acid molecule that includes a nucleic acid sequence having at least about 50%, preferably at least about 75%, and more preferably at least
15 about 85% homology with the corresponding region(s) of the nucleic acid sequence encoding the catalytic domain of an MEKK protein, or a homologue thereof. Also preferred is an MEKK protein nucleic acid molecule that includes a nucleic acid sequence having at least about 20%, preferably at
20 least about 30%, and more preferably at least about 40% homology with the corresponding region(s) of the nucleic acid sequence encoding the NH₂-terminal regulatory domain of an MEKK protein, or a homologue thereof. A particularly preferred nucleic acid sequence is a nucleic acid sequence
25 having at least about 50%, preferably at least about 75%, and more preferably at least about 85% homology with a nucleic acid sequence encoding the catalytic domain of an amino acid sequence represented by SEQ ID NO:2, SEQ ID

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NO:4, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:10. Another particularly preferred nucleic acid sequence is a nucleic acid sequence having at least about 20%, preferably at least about 30%, and more preferably at least about 40%
5 homology with a nucleic acid sequence encoding the NH₂-terminal regulatory domain of an amino acid sequence represented by SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:10.

Such nucleic acid molecules can be a full-length gene
10 and/or a nucleic acid molecule encoding a full-length protein, a hybrid protein, a fusion protein, a multivalent protein or a truncation fragment. More preferred nucleic acid molecules of the present invention comprise isolated nucleic acid molecules having a nucleic acid sequence as
15 represented by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:9.

Knowing a nucleic acid molecule of an MEKK protein of the present invention allows one skilled in the art to make copies of that nucleic acid molecule as well as to obtain
20 additional portions of MEKK protein-encoding genes (e.g., nucleic acid molecules that include the translation start site and/or transcription and/or translation control regions), and/or MEKK protein nucleic acid molecule homologues. Knowing a portion of an amino acid sequence of
25 an MEKK protein of the present invention allows one skilled in the art to clone nucleic acid sequences encoding such an MEKK protein.

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The present invention also includes nucleic acid molecules that are oligonucleotides capable of hybridizing, under stringent conditions, with complementary regions of other, preferably longer, nucleic acid molecules of the present invention that encode at least a portion of an MEKK protein, or a homologue thereof. A preferred oligonucleotide is capable of hybridizing, under stringent conditions, with a nucleic acid molecule that is capable of encoding at least a portion of an amino acid sequence represented by SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:10, or homologues thereof. A more preferred oligonucleotide is capable of hybridizing to a nucleic acid molecule having a nucleic acid sequence as represented by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and SEQ ID NO:9, or complements thereof.

Oligonucleotides of the present invention can be RNA, DNA, or derivatives of either. The minimal size of such oligonucleotides is the size required to form a stable hybrid between a given oligonucleotide and the complementary sequence on another nucleic acid molecule of the present invention. Minimal size characteristics are disclosed herein. The size of the oligonucleotide must also be sufficient for the use of the oligonucleotide in accordance with the present invention. Oligonucleotides of the present invention can be used in a variety of applications including, but not limited to, as probes to identify additional nucleic acid molecules, as primers to amplify or extend nucleic acid molecules or in therapeutic

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applications to inhibit, for example, expression of MEKK proteins by cells. Such therapeutic applications include the use of such oligonucleotides in, for example, antisense-, triplex formation-, ribozyme- and/or RNA drug-based technologies. The present invention, therefore, includes use of such oligonucleotides and methods to interfere with the production of MEKK proteins.

In one embodiment, an isolated MEKK protein of the present invention is produced by culturing a cell capable of expressing the protein under conditions effective to produce the protein, and recovering the protein. A preferred cell to culture is a recombinant cell that is capable of expressing the MEKK protein, the recombinant cell being produced by transforming a host cell with one or more nucleic acid molecules of the present invention. Transformation of a nucleic acid molecule into a cell can be accomplished by any method by which a nucleic acid molecule can be inserted into the cell. Transformation techniques include, but are not limited to, transfection, electroporation, microinjection, lipofection, adsorption, and protoplast fusion. A recombinant cell may remain unicellular or may grow into a tissue, organ or a multicellular organism. Transformed nucleic acid molecules of the present invention can remain extrachromosomal or can integrate into one or more sites within a chromosome of the transformed (i.e., recombinant) cell in such a manner that their ability to be expressed is retained.

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The present invention also includes a recombinant vector which includes at least one MEKK protein nucleic acid molecule of the present invention inserted into any vector capable of delivering the nucleic acid molecule into a host cell. Such a vector contains heterologous nucleic acid sequences, for example nucleic acid sequences that are not naturally found adjacent to MEKK protein nucleic acid molecules of the present invention. The vector can be either RNA or DNA, and either prokaryotic or eukaryotic, and is typically a virus or a plasmid. Recombinant vectors can be used in the cloning, sequencing, and/or otherwise manipulating of MEKK protein nucleic acid molecules of the present invention. One type of recombinant vector, herein referred to as a recombinant molecule and described in more detail below, can be used in the expression of nucleic acid molecules of the present invention. Preferred recombinant vectors are capable of replicating in the transformed cell.

Preferred nucleic acid molecules to insert into a recombinant vector includes a nucleic acid molecule that encodes at least a portion of an MEKK protein, or a homologue thereof. A more preferred nucleic acid molecule to insert into a recombinant vector includes a nucleic acid molecule encoding at least a portion of an amino acid sequence represented by SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 and/or SEQ ID NO:10, or homologues thereof. An even more preferred nucleic acid molecule to insert into a recombinant vector includes a nucleic acid

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molecule represented by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and/or SEQ ID NO:9, or complements thereof.

Suitable host cells for transforming a cell can include any cell capable of producing MEKK proteins of the present invention after being transformed with at least one nucleic acid molecule of the present invention. Host cells can be either untransformed cells or cells that are already transformed with at least one nucleic acid molecule.

10 Suitable host cells of the present invention can include bacterial, fungal (including yeast), insect, animal and plant cells. Preferred host cells include bacterial, yeast, insect and mammalian cells, with mammalian cells being particularly preferred.

15 A recombinant cell is preferably produced by transforming a host cell with one or more recombinant molecules, each comprising one or more nucleic acid molecules of the present invention operatively linked to an expression vector containing one or more transcription

20 control sequences. The phrase operatively linked refers to insertion of a nucleic acid molecule into an expression vector in a manner such that the molecule is able to be expressed when transformed into a host cell. As used herein, an expression vector is a DNA or RNA vector that is

25 capable of transforming a host cell and of effecting expression of a specified nucleic acid molecule. Preferably, the expression vector is also capable of replicating within the host cell. Expression vectors can

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be either prokaryotic or eukaryotic, and are typically viruses or plasmids. Expression vectors of the present invention include any vectors that function (i.e., direct gene expression) in recombinant cells of the present invention, including in bacterial, fungal, insect, animal, and/or plant cells. As such, nucleic acid molecules of the present invention can be operatively linked to expression vectors containing regulatory sequences such as promoters, operators, repressors, enhancers, termination sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell and that control the expression of nucleic acid molecules of the present invention. As used herein, a transcription control sequence includes a sequence which is capable of controlling the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include any transcription control sequence that can function in at least one of the recombinant cells of the present invention. A variety of such transcription control sequences are known to those skilled in the art. Preferred transcription control sequences include those which function in bacterial, yeast, and mammalian cells, such as, but not limited to, *tac*, *lac*, *trp*, *trc*, *oxy-pro*, *omp/lpp*, *rrnB*, bacteriophage lambda (λ) (such as λp_L and λp_R and fusions that include such promoters), bacteriophage T7,

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T7lac, bacteriophage T3, bacteriophage SP6, bacteriophage SP01, metallothionein, alpha mating factor, baculovirus, vaccinia virus, herpesvirus, poxvirus, adenovirus, simian virus 40, retrovirus actin, retroviral long terminal repeat, Rous sarcoma virus, heat shock, phosphate and nitrate transcription control sequences, as well as other sequences capable of controlling gene expression in prokaryotic or eukaryotic cells. Additional suitable transcription control sequences include tissue-specific promoters and enhancers as well as lymphokine-inducible promoters (e.g., promoters inducible by interferons or interleukins). Transcription control sequences of the present invention can also include naturally occurring transcription control sequences naturally associated with a DNA sequence encoding an MEKK protein.

Preferred nucleic acid molecules for insertion into an expression vector include nucleic acid molecules that encode at least a portion of an MEKK protein, or a homologue thereof. A more preferred nucleic acid molecule for insertion into an expression vector includes a nucleic acid molecule encoding at least a portion of an amino acid sequence represented by SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 and/or SEQ ID NO:10, or homologues thereof. An even more preferred nucleic acid molecule for insertion into an expression vector includes a nucleic acid molecule represented by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and/or SEQ ID NO:9, or complements thereof.

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Expression vectors of the present invention may also contain fusion sequences which lead to the expression of inserted nucleic acid molecules of the present invention as fusion proteins. Inclusion of a fusion sequence as part of an MEKK nucleic acid molecule of the present invention can enhance the stability during production, storage and/or use of the protein encoded by the nucleic acid molecule. Furthermore, a fusion segment can function as a tool to simplify purification of an MEKK protein, such as to enable purification of the resultant fusion protein using affinity chromatography. A suitable fusion segment can be a domain of any size that has the desired function (e.g., increased stability and/or purification tool). It is within the scope of the present invention to use one or more fusion segments. Fusion segments can be joined to amino and/or carboxyl termini of an MEKK protein. Linkages between fusion segments and MEKK proteins can be constructed to be susceptible to cleavage to enable straight-forward recovery of the MEKK proteins. Fusion proteins are preferably produced by culturing a recombinant cell transformed with a fusion nucleic acid sequence that encodes a protein including the fusion segment attached to either the carboxyl and/or amino terminal end of an MEKK protein.

A recombinant cell of the present invention includes any cells transformed with at least one of any nucleic acid molecule of the present invention. A preferred recombinant cell is a cell transformed with at least one nucleic acid molecule that encodes at least a portion of an MEKK

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protein, or a homologue thereof. A more preferred recombinant cell is transformed with at least one nucleic acid molecule that is capable of encoding at least a portion of an amino acid sequence represented by SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 SEQ ID NO:8 and/or SEQ ID NO:10, or homologues thereof. An even more preferred recombinant cell is transformed with at least one nucleic acid molecule represented by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and/or SEQ ID NO:9, or complements thereof. Particularly preferred recombinant cells include mammalian cells involved in a disease transformed with at least one of the aforementioned nucleic acid molecules.

It may be appreciated by one skilled in the art that use of recombinant DNA technologies can improve expression of transformed nucleic acid molecules by manipulating, for example, the number of copies of the nucleic acid molecules within a host cell, the efficiency with which those nucleic acid molecules are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of nucleic acid molecules of the present invention include, but are not limited to, operatively linking nucleic acid molecules to high-copy number plasmids, integration of the nucleic acid molecules into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers),

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substitutions or modifications of translational control signals (e.g., ribosome binding sites, Shine-Dalgarno sequences), modification of nucleic acid molecules of the present invention to correspond to the codon usage of the host cell, deletion of sequences that destabilize transcripts, and use of control signals that temporally separate recombinant cell growth from recombinant protein production during fermentation. The activity of an expressed recombinant protein of the present invention may be improved by fragmenting, modifying, or derivatizing the resultant protein.

As used herein, amplifying the copy number of a nucleic acid sequence in a cell can be accomplished either by increasing the copy number of the nucleic acid sequence in the cell's genome or by introducing additional copies of the nucleic acid sequence into the cell by transformation. Copy number amplification is conducted in a manner such that greater amounts of enzyme are produced, leading to enhanced conversion of substrate to product. For example, recombinant molecules containing nucleic acids of the present invention can be transformed into cells to enhance enzyme synthesis. Transformation can be accomplished using any process by which nucleic acid sequences are inserted into a cell. Prior to transformation, the nucleic acid sequence on the recombinant molecule can be manipulated to encode an enzyme having a higher specific activity.

In accordance with the present invention, recombinant cells can be used to produce an MEKK protein of the present

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invention by culturing such cells under conditions effective to produce such a protein, and recovering the protein. Effective conditions to produce a protein include, but are not limited to, appropriate media, 5 bioreactor, temperature, pH and oxygen conditions that permit protein production. An appropriate, or effective, medium refers to any medium in which a cell of the present invention, when cultured, is capable of producing an MEKK protein. Such a medium is typically an aqueous medium 10 comprising assimilable carbohydrate, nitrogen and phosphate sources, as well as appropriate salts, minerals, metals and other nutrients, such as vitamins. The medium may comprise complex nutrients or may be a defined minimal medium.

Cells of the present invention can be cultured in 15 conventional fermentation bioreactors, which include, but are not limited to, batch, fed-batch, cell recycle, and continuous fermentors. Culturing can also be conducted in shake flasks, test tubes, microtiter dishes, and petri plates. Culturing is carried out at a temperature, pH and 20 oxygen content appropriate for the recombinant cell. Such culturing conditions are well within the expertise of one of ordinary skill in the art.

Depending on the vector and host system used for production, resultant MEKK proteins may either remain 25 within the recombinant cell or be secreted into the fermentation medium. The phrase "recovering the protein" refers simply to collecting the whole fermentation medium containing the protein and need not imply additional steps

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of separation or purification. MEKK proteins of the present invention can be purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange
5 chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, chromatofocusing and differential solubilization.

In addition, an MEKK protein of the present invention
10 can be produced by isolating the MEKK protein from cells expressing the MEKK protein recovered from an animal. For example, a cell type, such as T cells, can be isolated from the thymus of an animal. MEKK protein can then be isolated from the isolated T cells using standard techniques
15 described herein.

The present invention also includes a method to identify compounds capable of regulating signals initiated from a receptor on the surface of a cell, such signal regulation involving in some respect, MEKK protein. Such
20 a method comprises the steps of: (a) contacting a cell containing an MEKK protein with a putative regulatory compound; (b) contacting the cell with a ligand capable of binding to a receptor on the surface of the cell; and (c) assessing the ability of the putative regulatory compound
25 to regulate cellular signals by determining activation of a member of an MEKK-dependent pathway of the present invention. A preferred method to perform step (c) comprises measuring the phosphorylation of a member of an

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MEKK-dependent pathway. Such measurements can be performed using immunoassays having antibodies specific for phosphotyrosines, phosphoserines and/or phosphothreonines. Another preferred method to perform step (c) comprises
5 measuring the ability of the MEKK protein to phosphorylate MEK protein and/or JEK protein using methods described herein.

In another embodiment, a method to identify compounds capable of regulating signal transduction in a cell can
10 comprise the steps of: (a) contacting a putative inhibitory compound with an MEKK protein to form a reaction mixture; (b) contacting the reaction mixture with MEK protein; and (c) assessing the ability of the putative inhibitory compound to inhibit phosphorylation of the MEK protein by
15 the MEKK protein. The results obtained from step (c) can be compared with the ability of a putative inhibitory compound to inhibit the ability of Raf protein to phosphorylate MEK protein, to determine if the compound can selectively regulate signal transduction involving MEKK
20 protein independent of Raf protein. MEKK, MEK and Raf proteins used in the foregoing methods can be recombinant proteins or naturally-derived proteins.

Moreover, one can determine whether the site of inhibitory action along a particular signal transduction
25 pathway involves both Raf and MEKK proteins by carrying out experiments set forth above (i.e., see discussion on MEKK-dependent pathways).

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Another aspect of the present invention includes a kit to identify compounds capable of regulating signals initiated from a receptor on the surface of a cell, such signals involving in some respect, MEKK protein. Such kits
5 include: (a) at least one cell containing MEKK protein; (b) a ligand capable of binding to a receptor on the surface of the cell; and (c) a means for assessing the ability of a putative regulatory compound to alter phosphorylation of the MEKK protein. Such a means for detecting
10 phosphorylation include methods and reagents known to those of skill in the art, for example, phosphorylation can be detected using antibodies specific for phosphorylated amino acid residues, such as tyrosine, serine and threonine. Using such a kit, one is capable of determining, with a
15 fair degree of specificity, the location along a signal transduction pathway of particular pathway constituents, as well as the identity of the constituents involved in such pathway, at or near the site of regulation.

In another embodiment, a kit of the present invention
20 can includes: (a) MEKK protein; (b) MEK protein; and (c) a means for assessing the ability of a putative inhibitory compound to inhibit phosphorylation of the MEK protein by the MEKK protein. A kit of the present invention can further comprise Raf protein and a means for detecting the
25 ability of a putative inhibitory compound to inhibit the ability of Raf protein to phosphorylate the MEK protein.

Another aspect of the present invention relates to the treatment of an animal having a medical disorder that is

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subject to regulation or cure by manipulating a signal transduction pathway in a cell involved in the disorder. Such medical disorders include disorders which result from abnormal cellular growth or abnormal production of secreted cellular products. In particular, such medical disorders include, but are not limited to, cancer, autoimmune disease, inflammatory responses, allergic responses and neuronal disorders, such as Parkinson's disease and Alzheimer's disease. Preferred cancers subject to treatment using a method of the present invention include, but are not limited to, small cell carcinomas, non-small cell lung carcinomas with overexpressed EGF receptors, breast cancers with overexpressed EGF or Neu receptors, tumors having overexpressed growth factor receptors of established autocrine loops and tumors having overexpressed growth factor receptors of established paracrine loops. According to the present invention, the term treatment can refer to the regulation of the progression of a medical disorder or the complete removal of a medical disorder (e.g., cure). Treatment of a medical disorder can comprise regulating the signal transduction activity of a cell in such a manner that a cell involved in the medical disorder no longer responds to extracellular stimuli (e.g., growth factors or cytokines), or the killing of a cell involved in the medical disorder through cellular apoptosis.

One aspect of the present invention involves the recognition that an MEKK protein of the present invention is capable of regulating the homeostasis of a cell by

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regulating cellular activity such as cell growth cell death, and cell function (e.g., secretion of cellular products). Such regulation, in most cases, is independent of Raf, however, as discussed above (and as shown in Fig. 2), some pathways capable of regulation by MEKK protein may be subject to upstream regulation by Raf protein. Therefore, it is within the scope of the present invention to either stimulate or inhibit the activity of Raf protein and/or MEKK protein to achieve desired regulatory results.

Without being bound by theory, it is believed that the regulation of Raf protein and MEKK protein activity at the divergence point from Ras protein (see Fig. 2) can be controlled by a "2-hit" mechanism. For example, a first "hit" can comprise any means of stimulating Ras protein, thereby stimulating a Ras-dependent pathway, including, for example, contacting a cell with a growth factor which is capable of binding to a cell surface receptor in such a manner that Ras protein is activated. Following activation of Ras protein, a second "hit" can be delivered that is capable of increasing the activity of JNK activity compared with MAPK activity, or vice versa. A second "hit" can include, but is not limited to, regulation of JNK or MAPK activity by compounds capable of stimulating or inhibiting the activity of MEKK, JEK, Raf and/or MEK. For example, compounds such as protein kinase C or phospholipase C kinase, can provide the second "hit" needed to drive the divergent Ras-dependent pathway down the MEKK-dependent

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thereof. A more preferred regulation scheme involves increasing the concentration of proteins including MEKK, JEK, JNK, Jun, ATF-2, and Myc, and combinations thereof. Another more preferred regulation scheme involves
5 decreasing the concentration of proteins including Raf, MEK, MAPK, and TCF, and combinations thereof. It is also within the scope of the present invention that the regulation of protein concentrations in two or more of the foregoing regulation schemes can be combined to achieve an
10 optimal apoptotic effect in a cell.

A preferred method for increasing the concentration of a protein in a regulation scheme of the present invention includes, but is not limited to, increasing the copy number of a nucleic acid sequence encoding such protein within a
15 cell, improving the efficiency with which the nucleic acid sequence encoding such protein is transcribed within a cell, improving the efficiency with which a transcript is translated into such a protein, improving the efficiency of post-translational modification of such protein, contacting
20 cells capable of producing such protein with anti-sense nucleic acid sequences, and combinations thereof.

In a preferred embodiment of the present invention, the homeostasis of a cell is controlled by regulating the apoptosis of a cell. A suitable method for regulating the
25 apoptosis of a cell is to regulate the activity of an MEKK-dependent pathway in which the MEKK protein regulates the pathway substantially independent of Raf. A particularly preferred method for regulating the apoptosis of a cell

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comprises increasing the concentration of MEKK protein by contacting a cell with a nucleic acid molecule encoding an MEKK protein that possesses unregulated kinase activity. A preferred nucleic acid molecule with which to contact a cell includes a nucleic acid molecule encoding an MEKK protein represented by SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:10, and combinations thereof. A more preferred nucleic acid molecule with which to contact a cell includes a nucleic acid molecule encoding a truncated MEKK protein having only the kinase catalytic domain (i.e., no regulatory domain) of an MEKK protein represented by SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 and/or SEQ ID NO:10. An even more preferred nucleic acid molecule with which to contact a cell includes a nucleic acid molecule including MEKK1₃₅₂₋₆₇₂, MEKK2₃₅₂₋₆₁₉, MEKK3₃₅₈₋₆₂₆, MEKK4₈₁₁₋₁₁₉₅, MEKK5₈₆₃₋₁₂₄₇, and combinations thereof. Again, suitable variations of MEKK proteins described herein comprise those proteins encoded by a nucleic acid molecule that are able to hybridize to any of the above sequences under stringent conditions.

It is within the scope of the invention that the foregoing method can further comprise the step of decreasing the activity of MEK protein in the cell by contacting the cell with a compound capable of inhibiting MEK activity. Such compounds can include: peptides capable of binding to the kinase domain of MEK in such a manner that phosphorylation of MAPK protein by the MEK protein is inhibited; and/or peptides capable of binding to a portion

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pathway in such a manner that JNK is preferentially activated over MAPK.

One embodiment of the present invention comprises a method for regulating the homeostasis of a cell comprising

5 regulating the activity of an MEKK-dependent pathway relative to the activity of a Raf-dependent pathway in the cell. As used herein, the term "homeostasis" refers to the tendency of a cell to maintain a normal state using intracellular systems such as signal transduction pathways.

10 Regulation of the activity of an MEKK-dependent pathway includes increasing the activity of an MEKK-dependent pathway relative to the activity of a Raf-dependent pathway by regulating the activity of a member of an MEKK-dependent pathway, a member of a Raf-dependent pathway, and

15 combinations thereof, to achieve desired regulation of phosphorylation along a given pathway, and thus effect apoptosis. Preferred regulated members of an MEKK-dependent pathway or a Raf-dependent pathway to regulate include, but are not limited to, proteins including MEKK,

20 Raf, JEK, MEK, MAPK, JNK, TCF, ATF-2, Jun and Myc, and combinations thereof.

In one embodiment, the activity of a member of an MEKK-dependent pathway, a member of a Raf-dependent pathway, and combinations thereof, are regulated by

25 altering the concentration of such members in a cell. One preferred regulation scheme involves altering the concentration of proteins including MEKK, Raf, JEK, MEK, MAPK, JNK, TCF, Jun, ATF-2, and Myc, and combinations

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of a MAPK protein in such a manner that phosphorylation of the MAPK protein is inhibited.

In another embodiment, the activity of a member of an MEKK-dependent pathway, a member of a Raf-dependent pathway, and combinations thereof, can be regulated by directly altering the activity of such members in a cell. A preferred method for altering the activity of a member of an MEKK-dependent pathway, includes, but is not limited to, contacting a cell with a compound capable of directly interacting with a protein including MEKK, JEK, JNK, Jun, ATF-2, and Myc, and combinations thereof, in such a manner that the proteins are activated; and/or contacting a cell with a compound capable of directly interacting with a protein including Raf, MEK, MAPK, TCF protein, and combinations thereof in such a manner that the activity of the proteins are inhibited. A preferred compound with which to contact a cell that is capable of regulating a member of an MEKK-dependent pathway includes a peptide capable of binding to the regulatory domain of proteins including MEKK, JEK, JNK, Jun, ATF-2, and Myc, in which the peptide inhibits the ability of the regulatory domain to regulate the activity of the kinase domains of such proteins. Another preferred compound with which to contact a cell includes TNF α , growth factors regulating tyrosine kinases, hormones regulating G protein-coupled receptors and FAS ligand.

A preferred compound with which to contact a cell that is capable of regulating a member of a Raf-dependent

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pathway includes a peptide capable of binding to the kinase catalytic domain of a protein selected from the group consisting of Raf, MEK-1, MEK-2, MAPK, and TCF, in which the peptide inhibits the ability of the protein to be phosphorylated or to phosphorylate a substrate.

One aspect of the present invention relates to the recognition that an MEKK protein is capable of activating MAPK. MAPK is known to be involved in various cellular pathways in mammalian systems. MAPK is known to be involved in cellular mitogenesis, DNA synthesis, cell division and differentiation. MAPK is also recognized as being involved in the activation of oncogenes, such as *c-jun* and *c-myc*. While not bound by theory, the present inventor believes that MAPK is also intimately involved in various abnormalities having a genetic origin. MAPK is known to cross the nuclear membrane and is believed to be at least partially responsible for regulating the expression of various genes. As such, MAPK is believed to play a significant role in the instigation or progression of cancer, neuronal diseases, autoimmune diseases, allergic reactions, wound healing and inflammatory responses. The present inventor, by being first to identify nucleic acid sequences encoding MEKK, recognized that it is now possible to regulate the expression of MEKK, and thus regulate the activation of MAPK.

The present invention also includes a method for regulating the homeostasis of a cell comprising injecting an area of a subject's body with an effective amount of a

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naked plasmid DNA compound. A naked plasmid DNA compound comprises a nucleic acid molecule encoding an MEKK protein of the present invention, operatively linked to a naked plasmid DNA vector capable of being taken up by and
5 expressed in a recipient cell located in the body area. A preferred naked plasmid DNA compound of the present invention comprises a nucleic acid molecule encoding a truncated MEKK protein having deregulated kinase activity. Preferred naked plasmid DNA vectors of the present
10 invention include those known in the art. When administered to a subject, a naked plasmid DNA compound of the present invention transforms cells within the subject and directs the production of at least a portion of an MEKK protein or RNA nucleic acid molecule that is capable of
15 regulating the apoptosis of the cell.

A naked plasmid DNA compound of the present invention is capable of treating a subject suffering from a medical disorder including cancer, autoimmune disease, inflammatory responses, allergic responses and neuronal disorders, such
20 as Parkinson's disease and Alzheimer's disease. For example, a naked plasmid DNA compound can be administered as an anti-tumor therapy by injecting an effective amount of the plasmid directly into a tumor so that the plasmid is taken up and expressed by a tumor cell, thereby killing the
25 tumor cell. As used herein, an effective amount of a naked plasmid DNA to administer to a subject comprises an amount needed to regulate or cure a medical disorder the naked plasmid DNA is intended to treat, such mode of

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administration, number of doses and frequency of dose capable of being decided upon, in any given situation, by one of skill in the art without resorting to undue experimentation.

- 5 Therapeutic compounds for use with a treatment method of the present invention can further comprise suitable excipients. A therapeutic compound for use with a treatment method of the present invention can be formulated in an excipient that the subject to be treated can
- 10 tolerate. Examples of such excipients include water, saline, Ringer's solution, dextrose solution, Hank's solution, and other aqueous physiologically balanced salt solutions. Nonaqueous vehicles, such as fixed oils, sesame oil, ethyl oleate, or triglycerides may also be used.
- 15 Other useful excipients include suspensions containing viscosity enhancing agents, such as sodium carboxymethylcellulose, sorbitol, or dextran. Excipients can also contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability.
- 20 Examples of buffers include phosphate buffer, bicarbonate buffer and Tris buffer, while examples of preservatives include thimerosal, m- or o-cresol, formalin and benzyl alcohol. Standard formulations can either be liquid injectables or solids which can be taken up in a suitable
- 25 liquid as a suspension or solution for injection. Thus, in a non-liquid formulation, the excipient can comprise dextrose, human serum albumin, preservatives, etc., to

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which sterile water or saline can be added prior to administration.

In another embodiment, a therapeutic compound for use with a treatment method of the present invention can also
5 comprise a carrier. Carriers are typically compounds that increase the half-life of a therapeutic compound in the treated animal. Suitable carriers include, but are not limited to, liposomes, micelles, cells, polymeric controlled release formulations, biodegradable implants,
10 bacteria, viruses, oils, esters, and glycols. Preferred carriers include liposomes and micelles.

A therapeutic compound for use with a treatment method of the present invention can be administered to any subject having a medical disorder as herein described. Acceptable
15 protocols by which to administer therapeutic compounds of the present invention in an effective manner can vary according to individual dose size, number of doses, frequency of dose administration, and mode of administration. Determination of such protocols can be
20 accomplished by those skilled in the art without resorting to undue experimentation. An effective dose refers to a dose capable of treating a subject for a medical disorder as described herein. Effective doses can vary depending upon, for example, the therapeutic compound used, the
25 medical disorder being treated, and the size and type of the recipient animal. Effective doses to treat a subject include doses administered over time that are capable of regulating the activity, including growth, of cells

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involved in a medical disorder. For example, a first dose of a naked plasmid DNA compound of the present invention can comprise an amount of that causes a tumor to decrease in size by about 10% over 7 days when administered to a
5 subject having a tumor. A second dose can comprise at least the same the same therapeutic compound than the first dose.

Another aspect of the present invention includes a method for prescribing treatment for subjects having a
10 medical disorder as described herein. A preferred method for prescribing treatment comprises: (a) measuring the MEKK protein activity in a cell involved in the medical disorder to determine if the cell is susceptible to treatment using a method of the present invention; and (b) prescribing
15 treatment comprising regulating the activity of an MEKK-dependent pathway relative to the activity of a Raf-dependent pathway in the cell to induce the apoptosis of the cell. The step of measuring MEKK protein activity can comprise: (1) removing a sample of cells from a subject;
20 (2) stimulating the cells with a $TNF\alpha$; and (3) detecting the state of phosphorylation of JEK protein using an immunoassay using antibodies specific for phosphothreonine and/or phosphoserine.

The present invention also includes antibodies capable
25 of selectively binding to an MEKK protein of the present invention. Such an antibody is herein referred to as an anti-MEKK antibody. Polyclonal populations of anti-MEKK antibodies can be contained in an MEKK antiserum. MEKK

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antiserum can refer to affinity purified polyclonal antibodies, ammonium sulfate cut antiserum or whole antiserum. As used herein, the term "selectively binds to" refers to the ability of such an antibody to preferentially bind to MEKK proteins. Binding can be measured using a variety of methods known to those skilled in the art including immunoblot assays, immunoprecipitation assays, enzyme immunoassays (e.g., ELISA), radioimmunoassays, immunofluorescent antibody assays and immunoelectron microscopy; see, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press, 1989.

Antibodies of the present invention can be either polyclonal or monoclonal antibodies and can be prepared using techniques standard in the art. Antibodies of the present invention include functional equivalents such as antibody fragments and genetically-engineered antibodies, including single chain antibodies, that are capable of selectively binding to at least one of the epitopes of the protein used to obtain the antibodies. Preferably, antibodies are raised in response to proteins that are encoded, at least in part, by a MEKK nucleic acid molecule. More preferably antibodies are raised in response to at least a portion of an MEKK protein, and even more preferably antibodies are raised in response to either the amino terminus or the carboxyl terminus of an MEKK protein. Preferably, an antibody of the present invention has a

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single site binding affinity of from about 10^3M^{-1} to about 10^{12}M^{-1} for an MEKK protein of the present invention.

A preferred method to produce antibodies of the present invention includes administering to an animal an effective amount of an MEKK protein to produce the antibody and recovering the antibodies. Antibodies of the present invention have a variety of potential uses that are within the scope of the present invention. For example, such antibodies can be used to identify unique MEKK proteins and recover MEKK proteins.

Another aspect of the present invention comprises a therapeutic compound capable of regulating the activity of an MEKK-dependent pathway in a cell identified by a process, comprising: (a) contacting a cell with a putative regulatory molecule; and (b) determining the ability of the putative regulatory compound to regulate the activity of an MEKK-dependent pathway in the cell by measuring the activation of at least one member of said MEKK-dependent pathway. Preferred methods to measure the activation of a member of an MEKK-dependent pathway include measuring the transcription regulation activity of c-Myc protein, measuring the phosphorylation of a protein selected from the group consisting of MEKK, JEK, JNK, Jun, ATF-2, Myc, and combinations thereof.

The following examples are provided for the purposes of illustration and are not intended to limit the scope of the present invention.

EXAMPLES

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Example 1

This example describes the structural characterization of MEKK 1 protein.

A. MEKK 1 Nucleotide Sequence

5 MEKK 1 protein was cloned by the following method. Unique degenerate inosine oligodeoxynucleotides were designed to correspond to regions of sequence identity between the yeast *Stell* and *Byr2* genes. With primers and cDNA templates derived from polyadenylated RNA from NIH 3T3
10 cells, a polymerase chain reaction (PCR) amplification product of 320 base pairs (bp) was isolated. This 320 bp cDNA was used as a probe to identify an MEKK 1 cDNA of 3260 bp from a mouse brain cDNA library using standard methods in the art. The MEKK 1 nucleotide sequence was determined
15 by dideoxynucleotide sequencing of double-stranded DNA using standard methods in the art.

Referring to Table 6, based on the Kozak consensus sequence for initiation codons, the starting methionine can be predicted to occur at nucleotide 486. With this
20 methionine at the start, the cDNA encodes a protein of 672 amino acids, corresponding to a molecular size of 73 kD. There is another in-frame methionine at position 441, which does not follow the Kozak rule, but would yield a protein of 687 amino acid residues (74.6 kD). Also referring to
25 Table 6, 20% of the NH₂-terminal 400 amino acids are serine or threonine and there are only two tyrosines. Several potential sites of phosphorylation by protein kinase C are

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apparent in the NH₂-terminal region. The kinase catalytic domain is located in the COOH-terminal half of the MEKK 1.

B. Southern Blot Analysis of MEKK 1 Transcript

Equal amounts (20 µg) of total RNA were loaded onto
5 the gel as indicated by ethidium bromide staining. Blots
were probed with either a 320-bp cDNA fragment encoding a
portion of the MEKK kinase domain or an 858-bp fragment
encoding a portion of the NH₂ terminal region of MEKK using
standard methods in the art. Referring to Fig. 3A, a 7.8
10 kb mRNA was identified with probes derived from both the 5'
and 3' ends of the MEKK cDNA in several cell lines and
mouse tissues. The MEKK mRNA was highly expressed in mouse
heart and spleen, and in lower amounts in liver.

C. Southern Blot Analysis

15 Mouse genomic DNA (10 µg) was digested with either *Bam*
HI, *Hind III* or *Eco RI* and applied to gels using standard
methods in the art. Blots were probed with a 320-bp
fragment of the MEKK gene. Fig. 3B shows the appearance of
one band in the *Bam HI* and *Hind III* digests which indicates
20 that MEKK is encoded by one gene. The appearance of two
bands in the *Eco RI* digest indicates the likely presence of
an *Eco RI* site within an intron sequence spanned by the
probe.

D. Immunoblots Using Anti-MEKK Antibodies

25 Three polyclonal antisera were prepared using three
different antigens. A first polyclonal antiserum was
prepared using an antigen comprising a 15 amino acid
peptide DRPPSRELLKHPVER derived from the COOH-terminus of

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MEKK. NZW rabbits were immunized with the peptide and antisera was recovered using standard methods known in the art. This first polyclonal antiserum is hereinafter referred to as the DRPP antiserum.

5 A second polyclonal antiserum was produced using a DNA clone comprising an *MEKK* cDNA digested with *EcoR*I and *Pst*I, thereby creating a 1270 bp fragment that encodes the amino terminus of *MEKK*. This fragment was cloned into *pRSETC* to form the recombinant molecule *pMEKK*₁₋₃₆₉ comprising amino
10 acid residues 1 to 369 of *MEKK* 1. The *pMEKK*₁₋₃₆₉ recombinant molecule was expressed in *E. coli* and protein encoded by the recombinant molecule was recovered and purified using standard methods known in the art. NZW rabbits were immunized with the purified recombinant *MEKK*₁₋₃₆₉
15 protein and antisera was recovered using standard methods known in the art. This second polyclonal antiserum is hereinafter referred to as the *MEKK*₁₋₃₆₉ antiserum.

 A third polyclonal antiserum was produced using a DNA clone comprising an *MEKK* cDNA digested with *Pst* I and *Kpn* I, thereby creating a 1670 bp fragment that encodes the
20 catalytic domain of *MEKK*. This fragment was cloned into *pRSETC* to form the recombinant molecule *pMEKK*₃₇₀₋₇₃₈ comprising amino acid residues 370 to 738 of *MEKK* 1 (encoded by base pairs 1592-3260). The *pMEKK*₃₇₀₋₇₃₈
25 recombinant molecule was expressed in *E. coli* and protein encoded by the recombinant molecule was recovered and purified using standard methods known in the art. NZW rabbits were immunized with the purified recombinant

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MEKK1₃₇₀₋₇₃₈ protein and antisera was recovered using standard methods known in the art. This second polyclonal antiserum is hereinafter referred to as the MEKK1₃₇₀₋₇₃₈ antiserum.

The DRPP antiserum was used to probe Western Blots of soluble cellular protein derived from several rodent cell lines. Soluble cellular protein (100 µg) or recombinant MEKK COOH-terminal fusion protein (30 ng) was loaded onto a 10% Tris Glycine SDS-PAGE gel and the protein transferred to a nylon filter using methods standard in the art. The nylon filter was immunoblotted with affinity purified DRPP antiserum (1:300 dilution). Referring to Fig. 3C, a 78 kD immunoreactive protein was identified in the samples comprising protein from Pheochromocytoma (PC12), Rat 1a, and NIH 3T3 cells. A prominent 50 kD immunoreactive band was also commonly present but varied in intensity from preparation to preparation indicating the band is a proteolytic fragment. Visualization of both the 78 kD and 50 kD immunoreactive bands on immunoblots was inhibited by pre-incubation of the 15 amino acid peptide antigen with the affinity purified DRPP antiserum. The MEKK protein detected by immunoblotting is similar to the molecular size predicted from the open reading frame of the MEKK cDNA.

In a second immunoblot experiment, PC12 cells stimulated or not stimulated with EGF were lysed and resolved on 10% Tris Glycine SDS-PAGE gel as described above. MEKK proteins contained in the cell lysates were identified by immunoblot using affinity purified MEKK1₁₋₃₆₉ antiserum (1:300) using methods standard in the art.

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Referring to Fig. 4, MEKK 1 and two higher molecular weight proteins having MEKK activity, MEKK α and MEKK β , were identified using the affinity purified MEKK1₁₋₃₆₉ antiserum. MEKK 1, and not MEKK α and MEKK β , were identified using
5 the affinity purified MEKK1₁₋₃₆₉ antiserum.

Using the same procedure described above, two MEKK immunoreactive species of approximately 98 kD and 82 kD present in PC12, Rat1a, NIH3T3, and Swiss3T3 cell lysates were recognized by affinity purified MEKK1₁₋₃₆₉ antiserum as
10 shown in Fig. 5. It should be noted that the 98 kD MEKK protein described herein was originally identified as a 95 kD MEKK protein in the related PCT application (International application no. PCT/US94/04178). Subsequent Tris Glycine SDS-PAGE gel analysis has led to the
15 determination that the modification in molecular weight. Visualization of both of these proteins was inhibited by incubation of the affinity purified MEKK1₁₋₃₆₉ antiserum with purified recombinant MEKK1₁₋₃₆₉ fusion protein antigen. A single 98 kD MEKK protein was present in MEKK
20 immunoprecipitates, but not in immunoprecipitates using preimmune serum. More of the 98 kD MEKK was expressed in PC12 cells relative to fibroblast cell lines. Immunoblotting with antibodies that specifically recognize Raf-1 or Raf-B indicated that neither of these enzymes were
25 present as contaminants of MEKK immunoprecipitates. 98 kD MEKK in MEKK immunoprecipitates did not comigrate with Raf-1 or Raf-B in PC12 cell lysates and no cross-reactivity between MEKK and Raf antibodies was observed.

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Example 2

This example describes the isolation of nucleic acid sequences encoding MEKK 2, MEKK 3 and MEKK 4 protein.

5 PCR primers were designed based on the nucleotide sequence of MEKK 1. PCR amplification of fragments from DNA isolated from reverse transcriptase reactions of RNA isolated from PC12 and HL60 cells was conducted using standard techniques. The resultant PCR products were cloned into the pGEX cloning vector (Promega, Wisconsin) using
10 standard procedures and submitted to DNA sequence analysis using standard techniques.

Example 3

This example describes the expression of MEKK 1 protein in COS-1 cells to define its function in regulating
15 the signaling system that includes MAPK.

COS cells in 100-mm culture dishes were transfected with either the pCVMV5 expression vector alone (1 µg: control) or the pCVMV5 MEKK construct (1 µg: MEKK). After 48 hours, the cells were placed in serum-free medium
20 containing bovine serum albumin (0.1 percent) for 16 to 18 hours to induce quiescence. Cells were then treated with human EGF (30 ng/ml)(+EGF) or buffer (control) for 10 minutes, washed twice in cold phosphate buffered saline (PBS), and lysed in cell lysis buffer containing 50 mM β-
25 glycerophosphate (pH 7.2), 100 µM sodium vanadate, 2 mM MgCl₂, 1mM EGTA Triton X-100 (0.5 percent), leupeptin (2 µg/ml), aprotinin (2µg/ml), and 1 mM dithiothreitol (600 µl). After centrifugation for 10 minutes at maximum speed

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in a microfuge, COS cell lysates containing 0.5 to 1 mg of soluble protein were subjected to FPLC on a MONO Q column, and eluted fractions were assayed for MAPK activity according to the method described in Heasley et al., p. 545, 1992, *Mol. Biol. Cell*, Vol. 3.

Referring to Fig. 6A, when MEKK 1 was overexpressed in COS 1 cells, MAPK activity was four to five times greater than that in control cells transfected with plasmid lacking an MEKK 1 cDNA insert. The activation of MAPK occurred in COS cells deprived of serum and in the absence of any added growth factor. The activity of MAPK was similar to that observed after stimulation of control cells with EGF. Stimulation of COS cells transiently overexpressing MEKK with EGF resulted in only a slight increase in MAPK activity compared to that observed with MEKK expression alone.

To ensure that MEKK protein was present in the samples tested for MAPK activity, protein from cell lysates of the transfected COS 1 cells were immunoblotted with MEKK specific antiserum. Equal amounts (100 μ g) of soluble protein lysate from COS cells were placed on the gel for immunoblotting using the methods described in Example 1. The filters were immunoblotted using the affinity purified DRPP antiserum (1:300) and affinity purified MEKK₁₋₃₆₉ antiserum (1:300). Referring to Fig. 6B, the results indicate that expression of MEKK in cells transfected with vector encoding MEKK that were treated with or without EGF. Only the 50 kD MEKK immunoreactive fragment was detected in

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lysates from control COS cells using the DRPP antiserum. Transient expression of MEKK in COS cells yielded a predominant 82 kD band that was slightly larger than that observed in PC12, Rat 1a, or NIH 3T3 cells. Addition of
5 the 15 amino acid DRPP peptide antigen to the antiserum during immunoblotting prevented detection of all of the immunoreactive bands; these bands were not detected in extracts of control COS cells, an indication that they were derived from the expressed MEKK protein.

10 Example 4

This Example describes the expression of MEKK 1 in COS cells to test the ability of MEKK protein to activate MEK protein.

Recombinant MAPK was used to assay MEK activity in COS
15 cell lysates that had been fractionated by fast protein liquid chromatography (FPLC) on a Mono S column. A cDNA encoding p42 MAPK from *Xenopus laevis* was cloned into the pRSETB expression vector. This construct was used for expression in the LysS strain of *Escherichia coli* BL21(DE3)
20 of a p42 MAPK fusion protein containing a polyhistidine sequence at the NH₂-terminus. Cultures containing the expression plasmid were grown at 37°C to an optical density of 0.7 to 0.9 at 600 nM. Isopropyl-β-thiogalactopyranoside (0.5 mM) was added to induce fusion protein synthesis and
25 the cultures were incubated for 3 hours. The cells were then collected and lysed by freezing, thawing, and sonication. The lysate was centrifuged at 10,000g for 15 minutes at 4°C. The supernatant was then passed over a Ni²⁺

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charged Sepharose resin and the soluble recombinant MAPK was eluted in sodium phosphate buffer (pH 4.5). The purified recombinant MAPK was more than 80 percent pure. The purified recombinant MAPK served as a substrate for MEK and catalyzed the phosphorylation of a peptide consisting of residues 662 to 681 of the EGF receptor (EGFR⁶⁶²⁻⁶⁸¹).

Soluble cell lysates from COS cells transiently transfected with MEKK, mock-transfected (control), or mock-transfected and treated with EGF (30 ng/ml) (+EGF), were fractionated by FPLC on a Mono S column and endogenous MEK activity was measured. Endogenous MAPK eluted in fractions 2 to 4, whereas MEK was contained in fractions 9 to 13. For assaying endogenous MEK activity, cells were washed twice in cold PBS and lysed in 650 μ l of a solution containing 50 mM β -glycerophosphate, 10 mM 2-N-morpholinoethane-sulfonic acid (pH 6.0), 100 μ M sodium vanadate, 2 mM $MgCl_2$, 1 mM EGTA, Triton X-100 (0.5 percent), leupeptin (5 μ g/ml), aprotinin (2 μ g/ml), and 1 mM dithiothreitol. After centrifugation at maximum speed for 10 minutes in a microfuge, soluble cell lysates (1 to 2 mg of protein) were applied to a Mono S column equilibrated in elution buffer (50 mM β -glycerophosphate, 10 mM MES (pH 6.0), 100 μ M sodium vanadate, 2 mM $MgCl_2$, 1 mM EGTA, and 1 mM dithiothreitol. The column was washed with buffer (2 ml) and bound proteins were eluted with a 30ml linear gradient of 0 to 350 mM NaCl in elution buffer. A portion (30 μ l) of each fraction was assayed for MEK activity by mixing with buffer (25 mM β -glycerophosphate, 40 mM N-(2-

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hydroxyethyl)piperazine-N'-(2-ethanolsulfonic acid) (pH 7.2)
50 mM sodium vanadate, 10 mM MgCl₂, 100 μM γ-³²P-ATP (3000
to 4000 cpm/pmol), inhibitor protein-20 (IP-20;
TTYADFIASGRTGRRNAIHD; 25 μg/ml), 0.5 mM EGTA, recombinant
5 MAP kinase (7.5 μg/ml), and 200 μM EGFR⁶⁶²⁻⁶⁸¹) in a final
volume of 40 μl. After incubation at 30°C for 20 minutes,
the incorporation of γ-³²P-ATP into EGFR⁶⁶²⁻⁶⁸¹ was measured.
In this assay, the ability of each column fraction to
activate added recombinant MAPK was measured by the
10 incorporation of γ-³²P-ATP into the MAPK substrate, a
peptide derived from the EGF receptor (EGFR).

Referring to Fig. 7, the first peak of activity eluted
represents endogenous activated MAPK, which directly
phosphorylates the EGFR peptide substrate. The second peak
15 of activity represents the endogenous MEK in COS cells.

The activity of endogenous MEK activity was
characterized by fractionation of Mono S FPLC. COS cell
lysates were fractionated by FPLC on a Mono Q column to
partially purify the expressed MEKK. Purified recombinant
20 MEK-1 was then used as a substrate for MEKK in the presence
of γ-³²P-ATP to determine whether MEKK directly
phosphorylates MEK-1.

A cDNA encoding MEK-1 was obtained from mouse B cell
cDNA templates with the polymerase chain reaction and
25 oligodeoxynucleotide primers corresponding to portions of
the 5' coding region and 3' untranslated region of MEK-1.
The catalytically inactive MEK-1 was generated by site-
directed mutagenesis of Lys³⁴³ to Met. The wild-type MEK-1

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and catalytically inactive MEK-1 proteins were expressed in pRSETA as recombinant fusion proteins containing a polyhistidine sequence at their NH₂-termini.

Lysates from COS cells transfected with MEKK or mock-transfected (control) were subjected to FPLC on a Mono Q column as described above. Portions (20 μ l) of fractions containing MEKK were mixed with buffer containing 50 mM β -glycerophosphate (pH 7.2), 100 μ M sodium vanadate, 2 mM MgCl₂, 1mM EGTA, 50 μ M ATP, IP-20 (50 μ g/ml), and 10 μ l γ -³²P-ATP in a reaction volume of 40 μ l and incubated for 40 minutes in the presence (+) or absence (-) of recombinant, catalytically inactive MEK-1 (150 ng)(kinase-MEK-1). Reactions were stopped by the addition of 5 x SDS sample buffer (10 μ l), 1 x SDS buffer contains 2 percent SDS, 5 percent glycerol, 62.5 mM tris-HCl (pH 6.8), 5 percent β -mercaptoethanol, and 0.001 percent bromophenol blue. The samples were boiled for 3 minutes and subjected to SDS-PAGE and autoradiography.

Referring to Fig. 8A, autophosphorylated recombinant wild-type MEK-1 (WT MEK-1) comigrated with phosphorylated catalytically inactive MEK-1. Thus, MEKK was capable of phosphorylating MEK-1. Corresponding fractions of lysates from control cells, however, were not able to phosphorylate MEK-1.

25 Example 5

This example describes studies showing that the modified form of MEK-1 that was used in the phosphorylation

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assay of Example 4 did not autophosphorylate as does wild-type MEK-1.

Phosphorylation of catalytically inactive MEK-1 by MEKK was time dependent (Fig. 8B); MEKK was also phosphorylated. Fraction 22 from FPLC on a Mono Q column (20 μ l) was incubated with or without recombinant catalytically inactive MEK-1 (0.15 μ g) for the indicated times. Referring to Fig. 8B, phosphorylation of kinase MEK-1 and MEKK was visible after 5 minutes and maximal after about 20 minutes. The time-dependent increase in MEKK phosphorylation correlated with a decreased mobility of the MEKK protein during SDS-PAGE. Referring to Fig. 8C, immunoblotting demonstrated that the MEKK protein co-eluted (after FPLC on a Mono Q column) with the peak of activity (fraction 22) that phosphorylated MEK. The slowly migrating species of MEKK were also detected by immunoblotting. Thus, expression of MEKK appears to activate MAPK by activating MEK.

Example 6

This Example describes that the phosphorylation of MEK by overexpressed MEKK resulted in activation of MEK, recombinant wild-type MEK-1 and a modified form of MAPK that is catalytically inactive.

COS cell lysates were separated by Mono Q-FPLC and fractions containing MEKK were assayed for their ability to activate added wild-type MEK-1 such that it would phosphorylate catalytically inactive recombinant MAPK in the presence of γ -³²P-ATP. Lysates from COS cells

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transfected with MEKK or mock-transfected (control) were fractionated by FPLC on a Mono Q column and portions (20 μ l) of fractions containing MEKK were mixed with buffer. Each fraction was incubated in the presence (+) or absence (-) of purified recombinant wild-type MEK-1 (150 ng) and in the presence of purified recombinant, catalytically inactive (kinase⁻) MAPK (300 ng). Referring to Fig. 9A, fractions 20 to 24 from lysates of COS cells transfected with MEKK activated MEK-1. Thus, MEKK phosphorylated and activated MEK-1, leading to MAPK phosphorylation.

Example 7

This Example describes studies demonstrating that MEKK activated MEK directly, and not through the activation of one or more other kinases contained in the column fractions.

Overexpressed MEKK was immunoprecipitated from COS cell lysates with affinity purified MEKK₁₋₃₆₉ antiserum. Immunoprecipitated MEKK was resuspended in 10 to 15 μ l of PAN (10 mM piperazine-N, N'-bis-2-ethanesulfonic acid (Pipes) (pH 7.0), 100 mM NaCl, and aprotinin (20 μ g/ml) and incubated with (+) or without (-) catalytically inactive MEK-1 (150 ng) and 25 μ Ci of γ -³²P-ATP in 20 mM pipes (pH 7.0), 10 mM MnCl₂, and aprotinin (20 μ g/ml) in a final volume of 20 μ l for 15 minutes 30°C. Reactions were stopped by the addition of 5 x SDS sample buffer (5 μ l). The samples were boiled for 3 minutes and subjected to SDS-PAGE and autoradiography.

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Referring to Fig. 9B, MEKK phosphorylated catalytically inactive MEK-1, which comigrated with wild-type MEK-1 on SDS-PAGE. Several phosphorylated bands of overexpressed MEKK were detected in the immunoprecipitates.

5 These bands probably resulted from autophosphorylation of MEKK and corresponded to the forms of MEKK identified by immunoblotting of lysates from COS cells transfected with MEKK. Immunoprecipitates obtained with pre-immune serum contained no MEKK and did not phosphorylate MEK-1. Thus,

10 MEKK appears to directly phosphorylate MEK.

Taken together, the results from Examples 4 through 7 show that MEKK can phosphorylate and activate MEK, which in turn phosphorylates and activates MAPK.

Example 8

15 This Example demonstrates that Raf can also phosphorylate and activate MEK.

COS cells deprived of serum were stimulated with EGF, and Raf was immunoprecipitated with an antibody to the COOH-terminus of Raf-1. Cos cells were transiently

20 transfected with vector alone (control) or with the PCV/M5-MEKK construct (MEKK). Quiescent control cells were treated with or without human EGF (30 ng/ml) for 10 minutes and Raf was immunoprecipitated from cell lysates with an antibody to a COOH-terminal peptide from Raf.

25 Immunoprecipitated Raf was incubated with catalytically inactive MEK-1 (150 ng) and 25 μ l of γ -³²P-ATP. The immunoprecipitated Raf phosphorylated MEK-1 in the presence of γ -³²P-ATP (Fig. 10A). Little or no phosphorylation of

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MEK-1 by Raf was observed in immunoprecipitates of Raf from COS cells overexpressing MEKK. Treatment of COS cells overexpressing MEKK with EGF resulted in a similar degree of phosphorylation of MEK-1 by immunoprecipitated Raf (Fig. 10B). Cells transfected with MEKK and deprived of serum were treated with EGF, and Raf was immunoprecipitated and incubated with catalytically inactive MEK-1. Equal amounts of Raf were immunoprecipitated in each sample as demonstrated by immunoblotting with antibodies to Raf. The slowest migrating band represents an immunoprecipitated phosphoprotein that is unrelated to Raf or MEK-1. The amount of Raf in the immunoprecipitates from control cells and cells transfected with MEKK was similar as shown by subsequent SDS-PAGE and immunoblotting with the antibody to Raf. Thus, both MEKK and Raf can independently activate MEK.

Example 9

This Example describes the activation of a 98 kD MEKK protein isolated from PC12 cells in response to stimulation of cells containing MEKK protein by growth factors.

PC12 cells were deprived of serum by incubation in starvation media (DMEM, 0.1% BSA) for 18-20 hours and MEKK was immunoprecipitated from lysates containing equal amounts of protein from untreated controls or cells treated with EGF (30ng/ml) or NGF (100ng/ml) for 5 minutes with the above-described anti-MEKK antibodies specific for the NH₂-terminal portion of MEKK. Immunoprecipitated MEKK was resuspended in 8μl of PAN (10mM piperazine-N,N'-bis-2-

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ethanesulfonic acid (Pipes) (pH 7.0), 100mM NaCl, and aprotinin (20 μ g/ml)) and incubated with catalytically inactive MEK-1 (150ng) and 40 μ Ci of (γ -³²P)ATP in universal kinase buffer (20mM piperazine-N,N'-bis-2-ethanesulfonic acid (Pipes) (pH 7.0), 10mM MnCl₂, and aprotinin (20 μ g/ml)) in a final volume of 20 μ l for 25 minutes at 30°C. Reactions were stopped by the addition of 2X SDS sample buffer (20 μ l). The samples were boiled for 3 minutes and subjected to SDS-PAGE and autoradiography. Raf-B was immunoprecipitated from the same untreated and treated PC12 cell lysates as above with an antiserum to a COOH-terminal peptide of Raf-B (Santa Cruz Biotechnology, Inc.) and assayed similarly. Raf-1 was immunoprecipitated with an antiserum to the 12 COOH-terminal amino acids of Raf-1 (Santa Cruz Biotechnology, Inc.). Epidermal growth factor (EGF) treatment of serum starved PC12 cells resulted in increased MEKK activity.

Referring to Fig. 11, the results were obtained by measuring the phosphorylation of purified MEK-1 (a kinase inactive form) by immunoprecipitates of MEKK in *in vitro* kinase assays. NGF stimulated a slight increase in MEKK activity compared to control immunoprecipitates from untreated cells. Stimulation of MEKK activity by NGF and EGF was similar to Raf-B activation by these agents, although Raf-B exhibited a high basal activity. Activation of c-Raf-1 by NGF and EGF was almost negligible in comparison to that of MEKK or Raf-B.

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A timecourse of MEKK stimulation by EGF was established by immunoprecipitating MEKK or Raf-B protein from lysates of PC12 cells treated with EGF (30ng/ml) for 0, 1, 3, 5, 10, or 20 minutes and incubating the protein with catalytically inactive MEK-1 (150ng) and (γ -³²P)ATP as described above. Data represent the relative magnitude of the response for each timepoint as quantitated by phosphorimager analysis of radioactive gels from a typical experiment. A timecourse of EGF treatment indicated that MEKK activation reached maximal levels following 5 minutes and persisted for at least 30 minutes (Fig. 12). Raf-B exhibited a similar timecourse; peak activity occurred within 3-5 minutes following EGF treatment and was persistent for up to 20 minutes.

To further dissociate EGF-stimulated MEKK activity from that of Raf-B, Raf-B was immunodepleted from cell lysates prior to MEKK immunoprecipitation. Raf-B was pre-cleared from lysates of serum-starved PC12 cells which had been either treated or not treated with EGF (30ng/ml) for 5 minutes. Raf-B was pre-cleared two times using antisera to Raf-B or using preimmune IgG antisera as a control. The pre-cleared supernatant was then immunoprecipitated with either MEKK or Raf-B antisera and incubated with catalytically inactive MEK-1 and (γ -³²P)ATP as described in detail above. EGF-stimulated and unstimulated PC12 cell lysates were pre-cleared with either IgG or Raf-B antisera and then subjected to immunoprecipitation with MEKK antiserum or Raf-B antibodies. The results shown in Fig.

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13 indicate that pre-clearing with Raf-B resulted in a 60% diminution of Raf-B activity as measured by phosphorimager analysis of Raf-B *in vitro* kinase assays. EGF-stimulated MEKK activity was unaffected by Raf-B depletion, suggesting
5 that Raf-B is not a component of MEKK immunoprecipitates. At least 40% of the Raf-B activity is resistant to preclearing with Raf-B antibodies. Recombinant wild type MEKK over-expressed in COS cells readily autophosphorylates on serine and threonine residues and the amino-terminus of
10 MEKK is highly serine and threonine rich. MEKK contained in immunoprecipitates of PC12 cells were tested for selective phosphorylation of purified recombinant MEKK amino-terminal fusion protein in *in vitro* kinase assays.

Serum-starved PC12 cells were treated with EGF
15 (30ng/ml) for 5 minutes and equal amounts of protein from the same cell lysates were immunoprecipitated with either MEKK, Raf-B, or preimmune antiserum as a control. Immunoprecipitates were incubated with purified recombinant MEKK NH₂-terminal fusion protein (400ng) and (γ -³²P)ATP as
20 described above. The results shown in Fig. 14 indicate that MEKK immunoprecipitated from lysates of EGF-stimulated and unstimulated PC12 cells robustly phosphorylated the inert 50 kD MEKK NH₂-fusion protein, while Raf-B or preimmune immunoprecipitates from EGF-stimulated or
25 unstimulated cells did not use the MEKK NH₂-fusion protein as a substrate. Thus, the EGF-stimulated MEKK activity contained in MEKK immunoprecipitates is not due to contaminating Raf kinases.

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Example 10

This Example describes MEKK activity in FPLC Mono Q iono-exchange column fractions of PC12 cell lysates.

Cell lysates were prepared from EGF-stimulated PC12
5 cells. Portions (900 μ l) of 1 ml column fractions (1 to
525 mM NaCl gradient) were concentrated by precipitation
with trichloroacetic acid and loaded on gels as described
above. The gels were blotted and then immunoblotted with
MEKK specific antibody. The results are shown in Fig. 15A
10 indicate that 98 kD MEKK immunoreactivity eluted in
fractions 10 to 12. The peak of B-Raf immunoreactivity
eluted in fraction 14, whereas Raf-1 was not detected in
the eluates from the column. Portions (30 μ l) of each
fraction from the PC12 lysates of unstimulated control
15 cells or EGF-treated cells were assayed as described above
in buffer containing purified recombinant MEK-1 (150 ng) as
a substrate. The results shown in Fig. 15B indicate that
the peak of MEKK activity eluted in fractions 10 to 12 from
EGF-stimulated PC12 cells phosphorylated MEK, whereas
20 little MEK phosphorylation occurred in fractions from
unstimulated cells.

Example 11

This Example describes studies demonstrating that the
phosphorylation of both MEK-1 and the MEKK NH₂-terminal
25 fusion protein were due to the activity of the 98 kD PC12
cell MEKK.

Cell lysates prepared from EGF-stimulated and
unstimulated cells were fractionated by FPLC on a Mono-Q

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column to partially purify the endogenous MEKK. Lysates from unstimulated control PC12 cells or cells treated with EGF (30ng/ml) for 5 minutes were fractionated by FPLC on a Mono Q column, using a linear gradient of 0 to 525 mM NaCl.

5 A portion (30 μ l) of each even numbered fraction was mixed with buffer (20mM piperazine-N,N'-bis-2-ethanesulfonic acid (Pipes) (pH 7.0), 10mM MnCl₂, aprotinin (20 μ g/ml), 50mM β -glycerophosphate (pH 7.2), 1mM EGTA, IP-20 (50 μ g/ml), 50mM NaF, and 30 μ Ci (γ -³²P)ATP) containing purified recombinant

10 MEK-1 (150ng) as a substrate in a final volume of 40 μ l and incubated at 30°C for 25 minutes. Reactions were stopped by the addition of 2X SDS sample buffer (40 μ l), boiled and subjected to SDS-PAGE and autoradiography. The peak of MEKK activity eluted in fractions 10-12. Portions (30 μ l)

15 of each even numbered fraction from lysates of EGF-treated PC12 cells were mixed with buffer as described above except containing purified recombinant MEKK NH₂-terminal fusion protein (400ng) as a substrate instead of MEK-1. Purified recombinant kinase inactive MEK-1 or the MEKK NH₂-terminal

20 fusion protein were then used as substrates in the presence of (γ -³²P)ATP to determine whether 98 kD MEKK directly phosphorylates either substrate. Fractions 10-14 of lysate from PC12 cells treated with EGF phosphorylated MEK-1 while little MEK-1 phosphorylation occurred in untreated control

25 fractions. The MEKK NH₂-terminal fusion protein was also phosphorylated in the same fractions as was MEK-1, although the peak of activity was slightly broader (fractions 8-16).

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Referring to Fig. 16, immunoblotting of column fractions demonstrated that the 98 kD MEKK protein co-eluted with the peak of activity that phosphorylated either exogenously added kinase inactive MEK-1 or the 50 kD MEKK NH₂-terminal fusion protein. Portions (900 μ l) of even numbered column fractions were concentrated by precipitation with trichloroacetic acid and immunoblotted with MEKK antibody. The peak of immunoreactivity eluted in fractions 10-12.

10 Example 12

This Example describes the activation of MEK by a 98 kD MEKK.

98 kD MEKK was immunoprecipitated using the MEKK₁₋₃₆₉ antiserum described in Example 1 from untreated (-) or EGF-treated (+) PC12 cell lysates. The immunoprecipitates were incubated in the presence (+) or absence (-) of purified recombinant wild-type MEK (150 ng) and in the presence of purified recombinant catalytically inactive MAPK (300 ng) and (γ -³²P)ATP. The results shown in Fig. 17A indicate that immunoprecipitated MEKK from EGF-stimulated cells phosphorylated and activated MEK, leading to MAPK phosphorylation. No phosphorylation of MAPK occurred in the absence of added recombinant MEK. Immunoblotting demonstrated that there was no contaminating MAPK (Fig. 17B) or contaminating MEK (Fig. 17C) in the MEKK immunoprecipitates from the EGF-stimulated PC12 cells. Thus, phosphorylation and activation of MEK is due to EGF

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stimulation of MEKK activity measured in the immunoprecipitates.

Example 13

This Example describes whether 98 kD PC12 cell MEKK
5 and Raf-B require functional Ras proteins for growth factor mediated signalling.

Dominant negative Ha-ras(Asn 17) (N¹⁷Ras) was expressed in PC12 cells and EGF-stimulated MEKK or Raf-B activation was assayed in immunoprecipitates using kinase inactive
10 MEK-1 as a substrate. PC12 cells stably expressing dexamethasone inducible N¹⁷Ras were serum starved for 18-20 hours in media containing 0.1% BSA with or without 1 μ M dexamethasone and then untreated or treated with EGF (30ng/ml) for 5 minutes. Equal amounts of soluble protein
15 from cell lysates was immunoprecipitated with either MEKK or Raf-B antisera and incubated with purified recombinant catalytically inactive MEK-1 and (γ -³²P)ATP as described above. Expression of N¹⁷Ras was induced in PC12 clones stably transfected with the N¹⁷Ras gene by the addition of
20 dexamethasone to the starvation media. N¹⁷Ras expression inhibited the activation of MEKK by EGF as measured by its ability to phosphorylate kinase inactive MEK. EGF-mediated activation of Raf-B was also greatly reduced in N¹⁷Ras expressing PC12 cells compared to uninduced N¹⁷Ras
25 transfectants. Addition of dexamethasone to wild type PC12 cells had no effect on the magnitude of MEKK or Raf-B activation elicited by EGF. PC12 cell clones stably transfected with the N¹⁷Ras gene are less responsive to EGF-

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mediated activation of MEKK activity than are wild type PC12 cells. These results indicate that functional Ras is required for growth factor stimulated activation of both Raf-B and MEKK in PC12 cells, suggesting that Ras may mediate its effects on cell growth and differentiation through the activation of multiple protein kinase effectors from both the Raf and MEKK families. Thus, EGF stimulated a peak of MEKK activity within 5 minutes which persisted for at least 30 minutes following treatment, and was similar to the timecourse of Raf-B activation. Nerve growth factor (NGF) and the phorbol ester TPA also activated MEKK, although to a lesser degree than EGF. MEKK activity in immunoprecipitates or column fractions was dissociable from that of EGF-stimulated c-Raf-1 and Raf-B activities. Forskolin pretreatment abolished both MEKK and Raf-B activation by EGF, NGF, and TPA (Fig. 18). Both MEKK and Raf-B activation in response to EGF was inhibited by stable expression of dominant negative N¹⁷ Ras. These findings represent the first demonstration of Ras-dependent MEKK regulation by growth factors and suggest the emergence of a complex intracellular kinase network in which Ras may alternately couple between members of the Raf and MEKK families.

To determine whether the growth factor-mediated activation of 98 kD PC12 cell MEKK was inhibited by PKA, forskolin was used to elevate intracellular cAMP and activate PKA. Serum-starved PC12 cells were pretreated with or without forskolin (50 μ M) for 3 minutes to activate

-100-

protein kinase A and then with EGF (30ng/ml), NGF (100ng/ml), or TPA (200nM) for 5 minutes and MEKK was immunoprecipitated from equal amounts of soluble protein from cell lysates and incubated with purified recombinant catalytically inactive MEK-1 and (γ -³²P)ATP as described above. Raf-B activity was also assayed from the same cell lysates to test whether its regulation differed from that of MEKK. Raf-B was immunoprecipitated from the same cell lysates as described above and assayed for its ability to phosphorylate MEK-1 as described above. Forskolin pretreatment abolished the activation of both MEKK and Raf-B by EGF, NGF, and TPA, as measured by their ability to phosphorylate kinase-inactive MEK-1 (Fig. 18). Forskolin treatment alone had no appreciable effect on either kinase. These results demonstrate that in addition to Raf-1 and Raf-B, PKA activation inhibits growth factor stimulation of 98 kD PC12 cell MEKK, suggesting the existence of a common regulatory control point for PKA action which lies between or downstream of Ras and upstream or at the level of each of these three kinases.

Example 14

This Example describes the determination of whether a similar or distinct MEK activity is involved in activation of MAPK through G_i protein coupled receptors by measuring MEK activity in cell lysates from thrombin stimulated Rat 1a cells.

Thrombin stimulated cells exhibited a MEK activity which co-fractionated with the major MEK peak detected in

-101-

EGF stimulated cells. The magnitude of MEK activity from thrombin challenged cells was generally two to three-fold less than that observed with EGF stimulation, which correlates with the smaller MAPK response the present
5 inventors have observed in thrombin challenged cells.

Differential regulation of MEK in Rat 1a and NIH3T3 cells expressing *gip2*, *v-src*, *v-ras*, or *v-raf* led the present inventor to investigate the protein kinases that are putative regulators of MEK-1. Recently, it was shown
10 that Raf-1 can phosphorylate and activate MEK. Raf activation was assayed in the following manner. Cells were serum starved and challenged in the presence or absence of the appropriate growth factors, as described above. Serum starved Rat 1a cells were challenged with buffer alone or
15 with EGF and Raf was immunoprecipitated using an antibody recognizing the C terminus of Raf. Cells were lysed by scraping in ice cold RIPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1.0% Triton X 100, 10 mM sodium pyrophosphate, 25 mM sodium
20 glycerophosphate, 2mM sodium vanadate, 2.1 μ g/ml aprotinin) and were microfuged for 10 min to remove nuclei. The supernatants were normalized for protein content and precleared with protein A Sepharose prior to immunoprecipitation with rabbit antiserum to the C terminus
25 of Raf-1 and protein A Sepharose for 2-3 h at 4°C. The beads were washed twice with ice cold RIPA and twice with PAN (10 mM Pipes, pH 7.0, 100mM NaCl, 21 μ g/ml aprotinin). A portion of the immunoprecipitate was diluted with SDS

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sample buffer and used for immunoblot analysis. The remainder was resuspended in kinase buffer (20 mM Pipes pH 7.0, 10 mM MnCl_2 , 150 ng kinase-inactive MEK-1, 30 μCi γ - ^{32}P -ATP and 20 $\mu\text{g/ml}$ aprotinin) in a final volume of 50 μl for 5 30 min at 30°C. Wild type recombinant MEK-1 was autophosphorylated in parallel as a marker. Reactions were terminated by the addition of 12.5 μl 5X SDS sample buffer, boiled for 5 minutes and subjected to SDS-PAGE and autoradiography.

10 The immunoprecipitated Raf, in the presence of γ - ^{32}P -ATP, was able to phosphorylate MEK-1. The recombinant MEK-1 used in this assay was kinase inactive to ensure it did not autophosphorylate as is observed with wild type MEK-1. Little or no phosphorylation of MEK-1 by Raf was observed 15 in immunoprecipitates from control cells. EGF challenge clearly stimulated Raf catalyzed phosphorylation of MEK-1; in contrast, thrombin challenge of Rat 1a cells did not measurably activate Raf even though endogenous MEK was clearly activated. EGF stimulated Raf phosphorylation of 20 recombinant MEK-1 by approximately 2.6-fold over basal. Little phosphorylation of MEK by Raf was observed in Raf immunoprecipitates from Gip2 or v-Src expressing Rat 1a cells. EGF stimulation was still capable of activating Raf catalyzed phosphorylation of MEK-1 in these cell lines by 25 1.8 and 1.4-fold, respectively. The blunting of the EFG response in Gip2 and v-Src expressing cells is likely a result of desensitization of the EFG receptor upon constitutive activation of MAPK. The amount of Raf in the

-103-

immunoprecipitates was shown to be similar by subsequent SDS-PAGE and immunoblotting using Raf antibody. Since thrombin stimulation of MEK is two to three-fold over basal, at least a 1.5-fold stimulation of MEK phosphorylation is expected if Raf significantly contributed to MEK activation by this growth factor. This level of activation was detectable in the EGF stimulated G12 and v-Src expressing cells lines. Thus, it is unlikely that the failure to detect thrombin activation of Raf is due to the sensitivity of the assay. Thrombin stimulation of MAPK is maximal at 3 minutes. Stimulation of Rat 1a cells for 1 or 5 minutes with thrombin did not increase Raf activity.

In NIH3T3 cells, as in Rat 1a cells, EGF activates Raf approximately 2.7-fold, while thrombin does not. V-Raf expressing NIH3T3 cells showed no increase in MEK-1 phosphorylation. This result was unexpected since MEK was clearly activated in v-Raf expressing NIH3T3 cells. Both the p90 and p75 gag-raf fusion proteins in addition to c-Raf-1 were immunoprecipitated from v-Raf NIH3T3 cells by the antisera. P75gag-raf has been shown to exhibit protein kinase activity, but it is possible that the NH₂ terminal gag fusion protein sterically hinders Raf phosphorylation of recombinant MEK-1 in the *in vitro* assay system. Further studies will have to be done to measure v-Raf kinase activity. The results argue that activation of MEK cannot be accounted for exclusively by the activation of Raf. Additional regulatory kinases for MEK must exist which

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contribute to MEK activation in thrombin stimulated, G_i protein coupled pathways and in *gip2* and *v-src* transfected cells.

Example 15

5 This Example demonstrates the ability of a PPPSS-trunc and Nco1-trunc of MEKK protein to activate MAPK activity compared with full-length MEKK protein and a negative control protein.

10 The results shown in Fig. 19 indicate that the truncated MEKK molecules were more active than the full-length MEKK. Indeed, the truncated MEKK molecules were at least about 1.5 times more active than full-length MEKK protein. Thus, removal of the regulatory domain of MEKK
15 deregulates the activity of the catalytic domain resulting in improved enzyme activity.

Example 16

 This example describes the preferential activation of JNK by MEKK compared with Raf.

20 HeLa cells were transiently transfected with truncated MEKK₃₇₀₋₇₃₈ under control of an inducible mammary tumor virus promoter, together with epitope tagged JNK1 (described in detail in Derijard et al., p. 1028, 1994, *Cell*, Vol. 76). Other HeLa cells were also transiently transfected with truncated BXB-Raf under control of an inducible mammary
25 tumor virus promoter, together with epitope tagged JNK1 (Derijard et al., *ibid.*). The following day, MEKK₃₇₀₋₇₃₈ expression and BXB-Raf expression were induced by administration of dexamethasone (10 μ M) for 17 hours. Cell

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extracts were then prepared and assayed for JNK activity using an immune complex kinase assay (detailed in Derijard et al., *ibid.*). Phosphorylation was quantitated by phosphorimager analysis. The results shown in Fig. 20 indicate that MEKK stimulated about 30-fold to about 50-fold activation more JNK activity over unstimulated cells (basal) and about 15-fold to about 25-fold JNK activity over Raf stimulated cells.

Example 17

10 This example describes that the phosphorylation of c-Myc transactivation domain in response to MEKK expression activates MYC-GAL 4 transcriptional activity.

Two separate expression plasmids were constructed as follows. The expression plasmid pLNCX was ligated to a cDNA clone comprising c-myc (1-103) ligated to GAL4 (1-147) (Seth et al., pp. 23521-23524, 1993, *J. Biol. Chem.*, Vol. 266) to form the recombinant molecule pMYC-GAL 4. The expression plasmid UAS_c-TK Luciferase (Sadowski et al., pp. 563-564, 1988, *Nature*, Vol. 335) was transfected with either pMYC-GAL 4 or pLU-GAL into Swiss 3T3 cells using standard methods in the art to form recombinant cells herein referred to as LU/GAL cells. Recombinant control cells were also produced by transfecting in pGAL4-Control plasmids containing GAL4 (1-147) alone in the absence of c-myc (1-103).

LU/Gal cells were transfected with either pMEKK₃₇₀₋₇₃₈, pMEKK (encoding full-length MEKK₁₋₇₃₈), BXB-Raf, pMyc-Gal4, pCREB-Gal4 (encoding CREB₁₋₂₆₁ fused to Gal 4₁₋₁₄₇; Hoeffler et

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al., pp. 868-880, 1989, *Mol. Endocrinol.*, Vol. 3), pGal4, or CREB fusion protein referred to as GAL4.

The transfected cells were incubated overnight and then lysed using methods standard in the art. The
5 luciferase activity of each cell lysate was measure on a luminometer. The results shown in Fig. 21 indicate that MEKK is selectively capable of stimulating the phosphorylation of c-Myc transactivation domain in such a manner that the c-Myc domain is activated and induces
10 transcription of the transfected luciferase gene. In addition, the results indicate that MEKK does not stimulate CREB activation. Also, activated Raf is unable to stimulate Myc activation. A schematic representation of the activation mechanism of c-Myc protein by MEKK is shown
15 in Fig. 23.

Example 18

This Example describes the phosphorylation of p38 MAPK protein by MEKK 3 protein and not MEKK 1 protein.

COS cells were transfected with the expression plasmid
20 pCVM5 ligated to cDNA clones encoding either MEKK 1 or MEKK 3 protein, or a control pCVM5 plasmid lacking MEKK cDNA inserts. Forty-eight hours after transfection, the COS cells were lysed and the lysate fractionated on a Mono Q FPLC column using conditions described in Example 4. The
25 fractions were analyzed for tyrosine phosphorylation of MAP kinase-like enzymes using the kinase assay described in Example 4. Referring to Fig. 23, expression of MEKK 3 induces tyrosine phosphorylation of p38 MAPK and the p42

-107-

and p44 forms of MAPK. MEKK 1, however, only induces weak phosphorylation of p38 MAPK but does induce phosphorylation of p42 and p44 MAPK.

Example 19

5 This example describes MEKK-induced apoptosis.

Cells were prepared for the apoptosis studies as follows. Swiss 3T3 cells and REF52 cells were transfected with an expression plasmid encoding β -Galactosidase (β -Gal) detection of injected cells. One set of β -Gal
10 transfected cells were then microinjected with an expression vector encoding MEKK₃₇₀₋₇₃₈ protein. Another set of β -Gal transfected cells were then microinjected with an expression vector encoding truncated BXB-Raf protein.

A. Beauvericin-induced apoptosis

15 A first group of transfected Swiss 3T3 cells and REF52 cells were treated with 50 μ M beauvericin for 6 hours at 37°C. Beauvericin is a compound known to induce apoptosis in mammalian cells. A second group of cells were treated with a control buffer lacking beauvericin. The treated
20 cells were then fixed in paraformaldehyde and permeabilized with saponin using protocols standard in the art. The permeabilized cells were then labelled by incubating the cells with a fluorescein-labelled anti-tubulin antibody (1:500; obtained from GIBCO, Gaithersburg, MD) to detect
25 cytoplasmic shrinkage or 10 μ M propidium iodide (obtained from Sigma, St. Louis, MO) to stain DNA to detect nuclear condensation. The labelled cells were then viewed by differential fluorescent imaging using a Nikon Diaphot

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fluorescent microscope. Fig. 24 shows two fields of Swiss 3T3 cells and REF52 cells, one field representing cells treated with the control buffer and a second field representing cells treated with beauvericin. The cells treated with beauvericin demonstrated cytoplasmic shrinkage (monitored by the anti-tubulin antibodies) and nuclear condensation (monitored by the propidium iodide) characteristic of apoptosis.

B. MEKK-induced apoptosis

Swiss 3T3 cells and REF52 cells microinjected with a β -galactosidase expression plasmid, and an MEKK encoding plasmid or a BXB-Raf encoding plasmid, were treated and viewed using the method described above in Section A. An anti- β -Gal antibody (1:500, obtained from GIBCO, Gaithersburg MD) was used to detect injected cells. Referring to Fig. 25, microscopic analysis of REF52 cells indicated that the cells expressing MEKK protein underwent cytoplasmic shrinkage and nuclear condensation leading to apoptotic death. In contrast, cells expressing BXB-Raf protein displayed normal morphology and did not undergo apoptosis. Similarly, referring to Fig. 26, microscopic analysis of Swiss 3T3 cells indicated that the cells expressing MEKK protein underwent cytoplasmic shrinkage and nuclear condensation leading to apoptotic death. In contrast, cells expressing BXB-Raf protein displayed normal morphology and did not undergo apoptosis.

Fig. 27 shows 3 representative fields of RFE52 cells expressing MEKK protein which have undergone substantial

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cytoplasmic shrinkage and nuclear condensation compared with a control cell not expressing MEKK. Similarly, Fig. 28 shows 3 representative fields of Swiss 3T3 cells expressing MEKK protein which have undergone substantial cytoplasmic shrinkage and nuclear condensation compared with a control cell not expressing MEKK. Thus, MEKK and not Raf protein can induce apoptotic programmed cell death.

Example 20

This Example describes regulation of MAPK activity by both MEKK and Raf protein.

COS cells were prepared using the method described in Example 3. In addition, COS cells were transfected with the pCVMV5 Raf construct (1 μ g: Raf). FPLC MONO Q ion-exchange column fractions were prepared as described in Example 3 and assayed for MAPK activity according to the method described in Heasley et al., *ibid*.

Referring to Fig. 29, both MEKK and Raf overexpression in COS 1 cells resulted in similar levels of stimulation of MAPK activity over basal levels.

The foregoing description of the invention has been presented for purposes of illustration and description. Further, the description is not intended to limit the invention to the form disclosed herein. Consequently, variations and modifications commensurate with the above teachings, and the skill or knowledge in the relevant art are within the scope of the present invention. The preferred embodiment described herein above is further intended to explain the best mode known of practicing the

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invention and to enable others skilled in the art to
utilize the invention in various embodiments and with
various modifications required by their particular
applications or uses of the invention. It is intended that
5 the appended claims be construed to include alternate
embodiments to the extent permitted by the prior art.

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What is Claimed:

1. An isolated protein comprising at least a portion of an amino acid sequence encoded by a nucleic acid sequence that is capable of hybridizing under stringent conditions with a nucleic acid molecule encoding an amino acid sequence selected from the group consisting of SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:10.
2. A formulation comprising at least one isolated protein having at least a portion of an amino acid sequence encoded by a nucleic acid sequence that is capable of hybridizing under stringent conditions with a nucleic acid molecule encoding an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:10.
3. An isolated nucleic acid molecule capable of hybridizing under stringent conditions with a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and SEQ ID NO:9.
4. A recombinant molecule, comprising a nucleic acid molecule capable of hybridizing under stringent conditions with a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and SEQ ID NO:9, said nucleic acid molecule being operatively linked to an expression vector.
5. A recombinant cell transformed with a recombinant molecule, comprising a nucleic acid molecule operatively linked to an expression vector, said nucleic acid molecule comprising a nucleic acid sequence capable of hybridizing

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under stringent conditions with a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 and SEQ ID NO:9.

6. A method for regulating the homeostasis of a cell
5 comprising regulating the activity of an MEKK-dependent pathway relative to the activity of a Raf-dependent pathway in said cell.

7. A method to treat a medical disorder, comprising
10 regulating the activity of an MEKK-dependent pathway relative to the activity of a Raf-dependent pathway in a cell.

8. A method to inhibit a medical disorder selected
from the group consisting of tumorigenesis and
autoimmunity, comprising regulating the activity of an
15 MEKK-dependent pathway relative to the activity of a Raf-dependent pathway in a cell to inhibit the growth of said cell.

9. A therapeutic compound capable of regulating the
activity of an MEKK-dependent pathway in a cell identified
20 by a process, comprising:

- a) contacting a cell with a putative regulatory molecule; and
- b) determining the ability of said putative regulatory compound to regulate the activity of an MEKK-
25 dependent pathway in said cell by measuring the activation of at least one member of said MEKK-dependent pathway.

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10. A method to treat a disease, comprising administering to a patient an effective amount of a therapeutic compound comprising at least one regulatory molecule selected from the group consisting of a molecule
5 capable of decreasing the activity of a Raf-dependent pathway, a molecule capable of increasing the activity of an MEKK-dependent pathway, and combinations thereof, wherein said effective amount comprises an amount which results in the depletion of harmful cells involved in said
10 disease.

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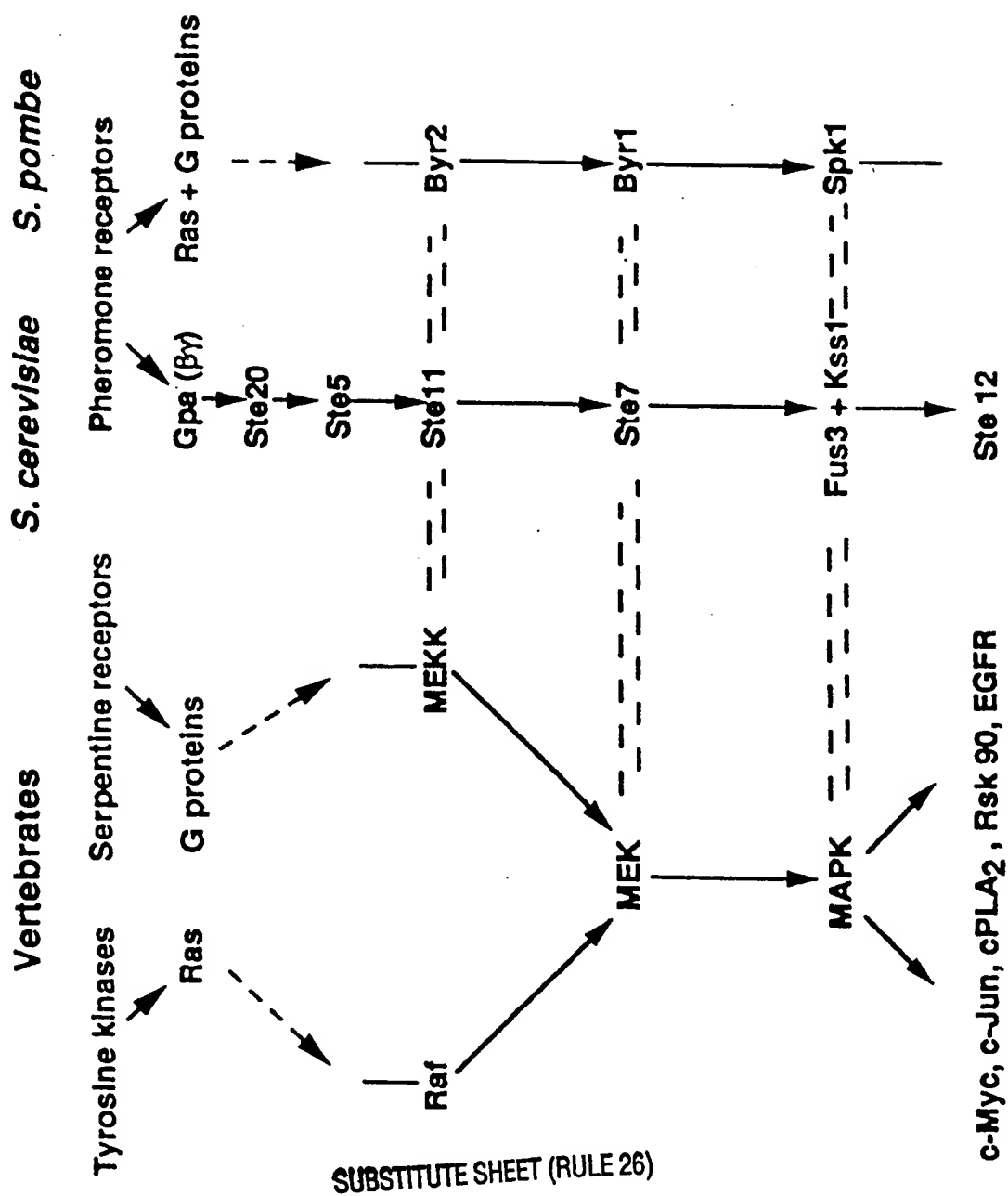


FIG. 1

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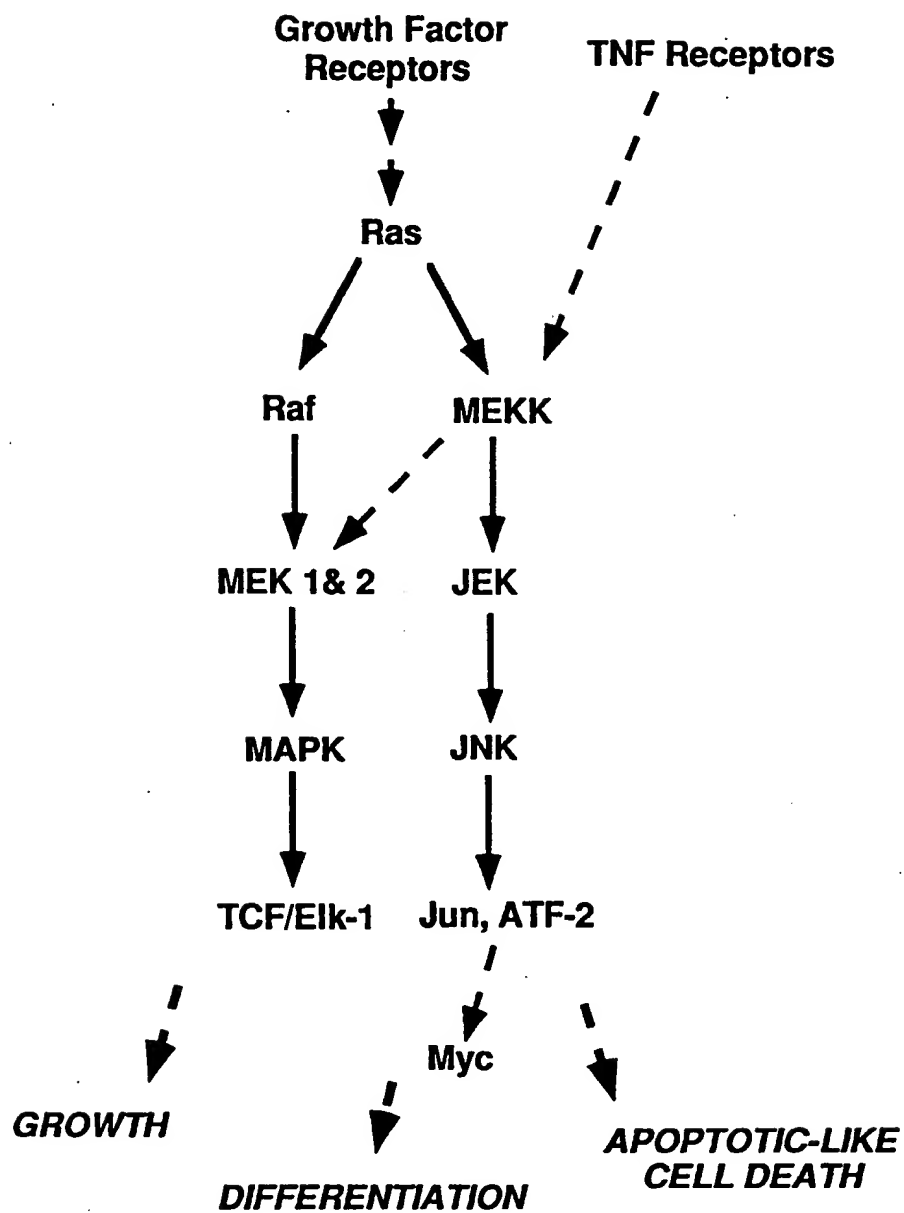


FIG. 2

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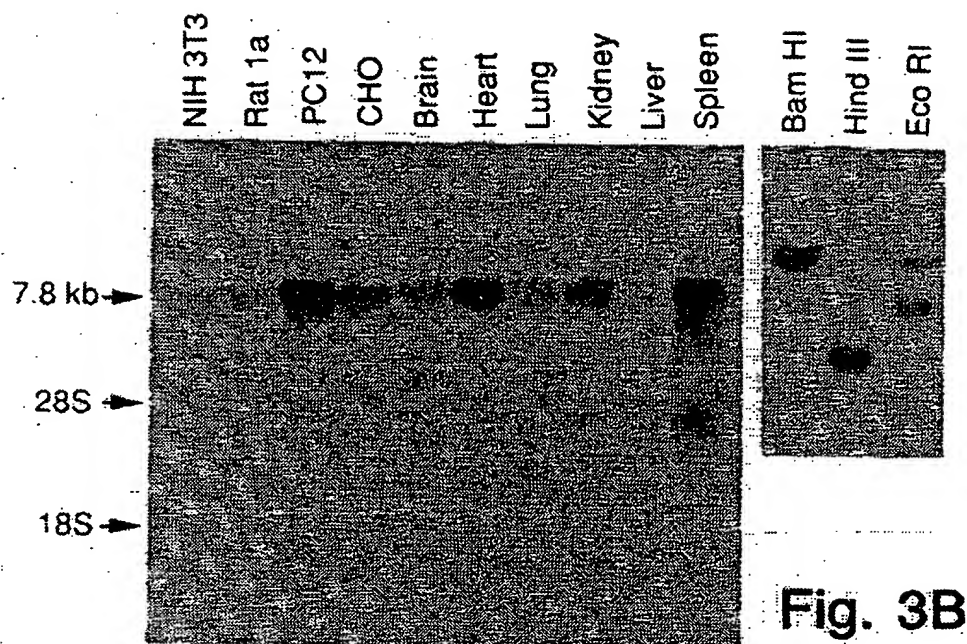


Fig. 3A

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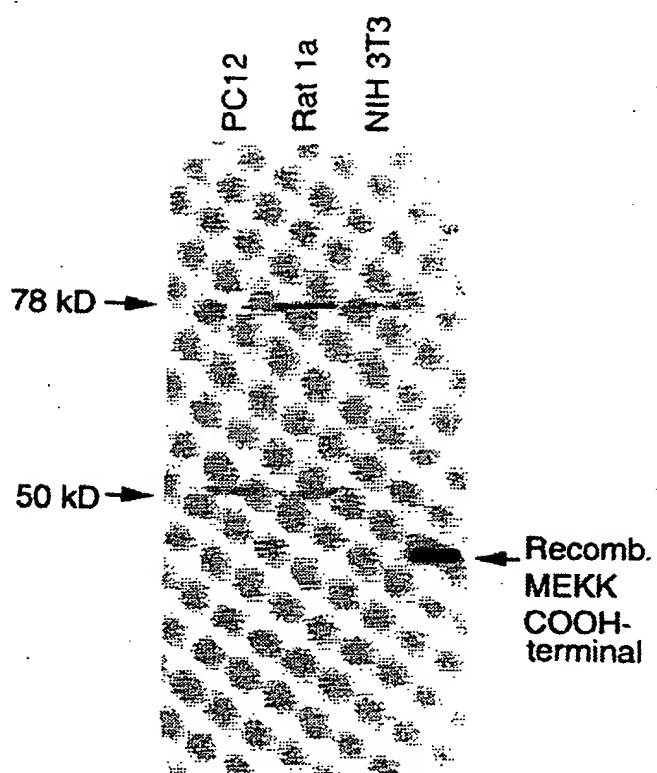


Fig. 3C

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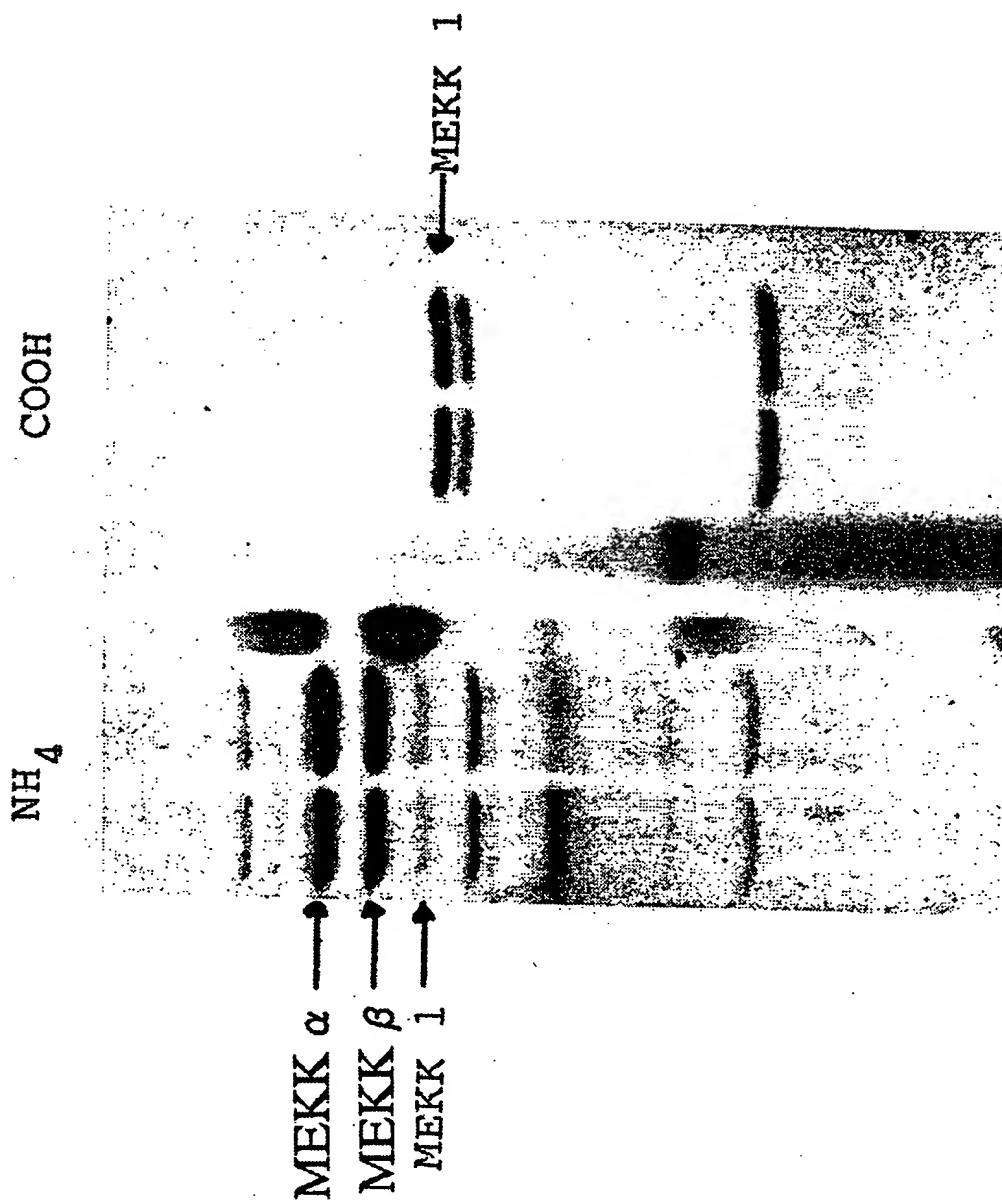


Fig. 4

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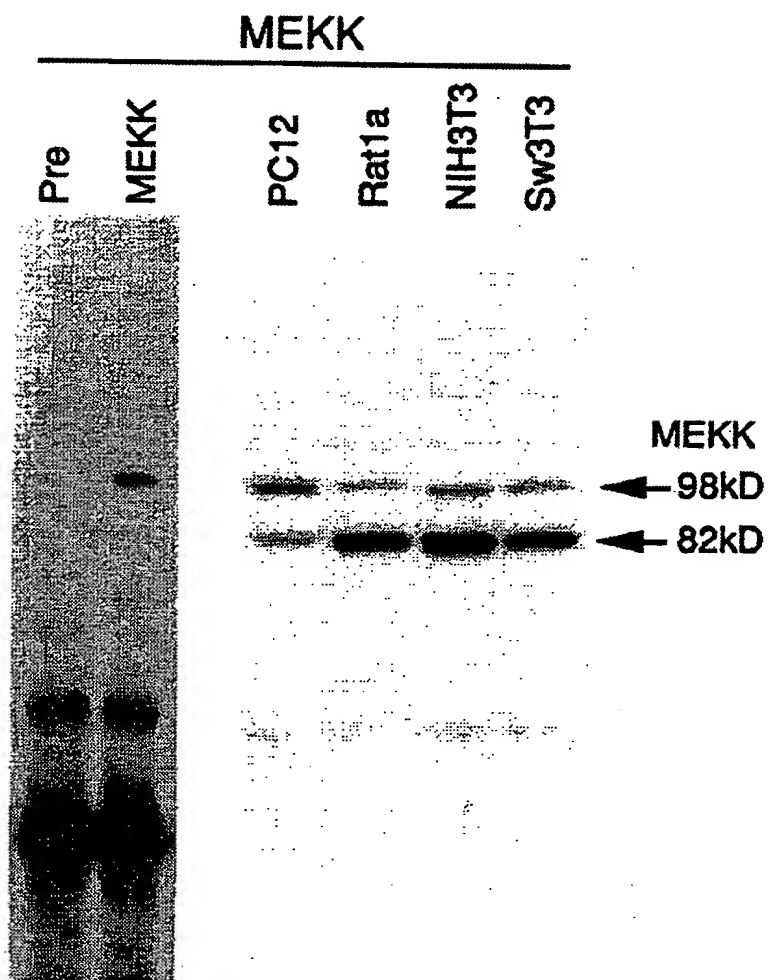


Fig. 5A

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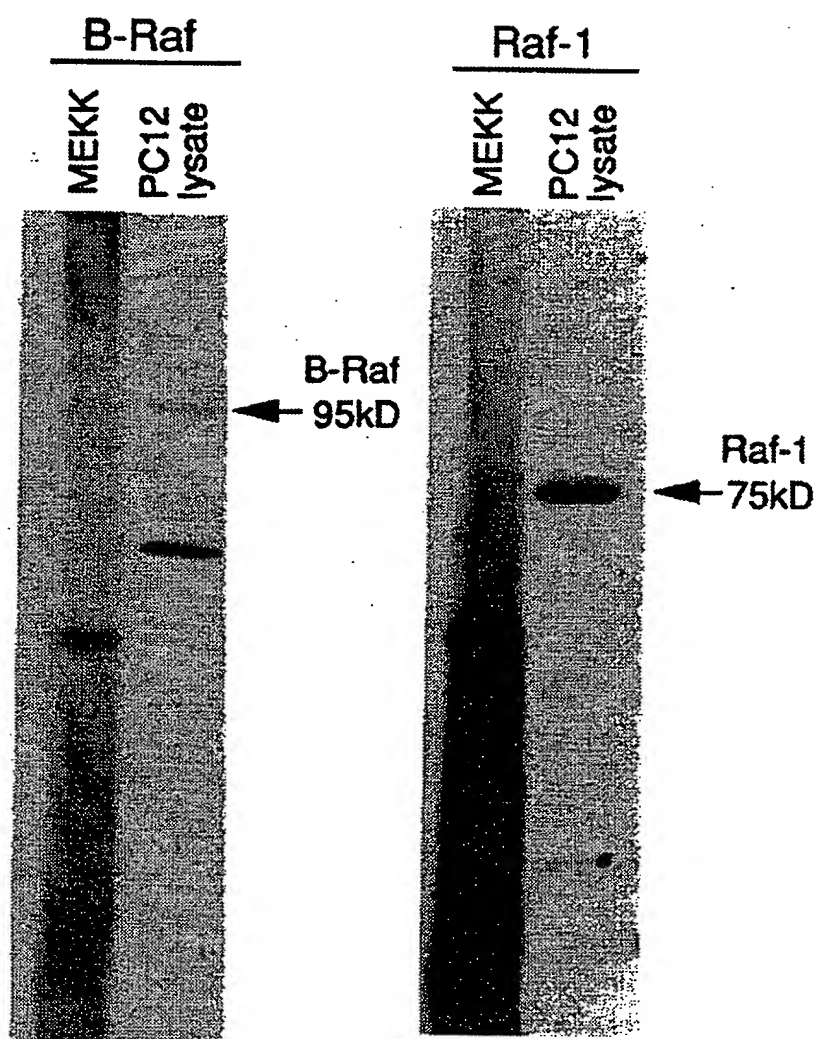


Fig. 5B

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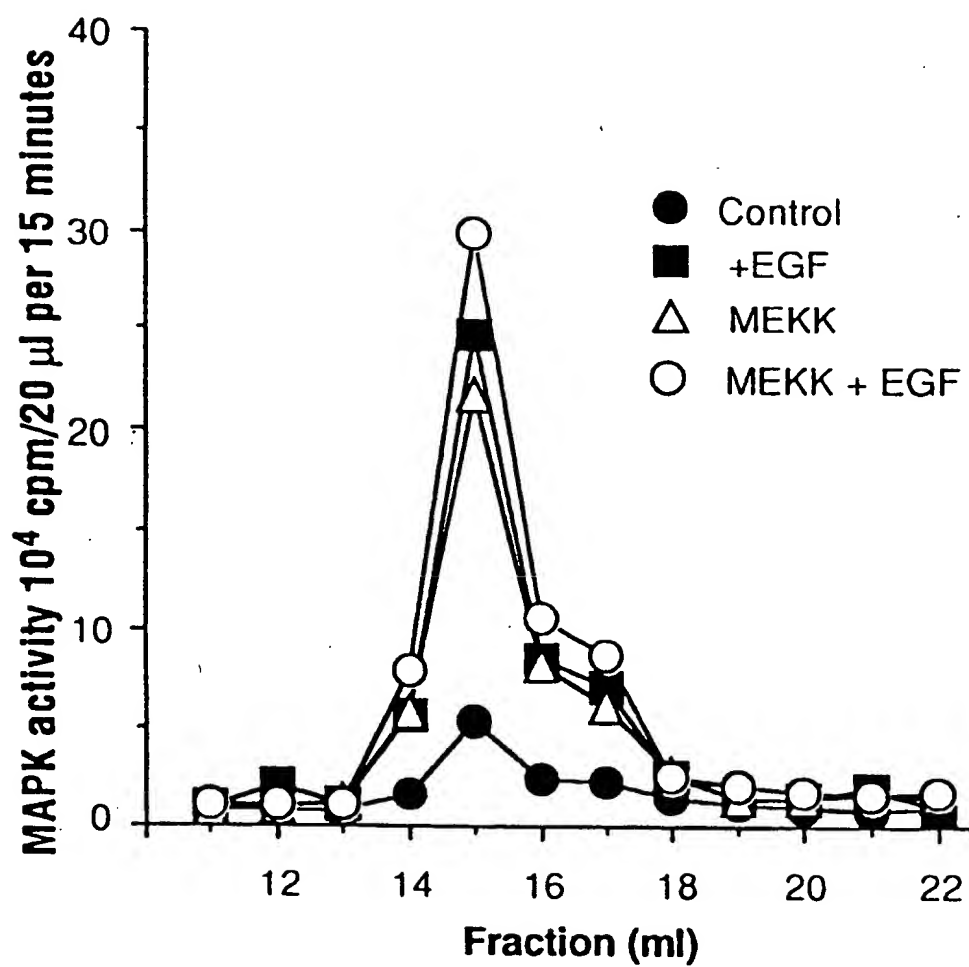


FIG. 6A

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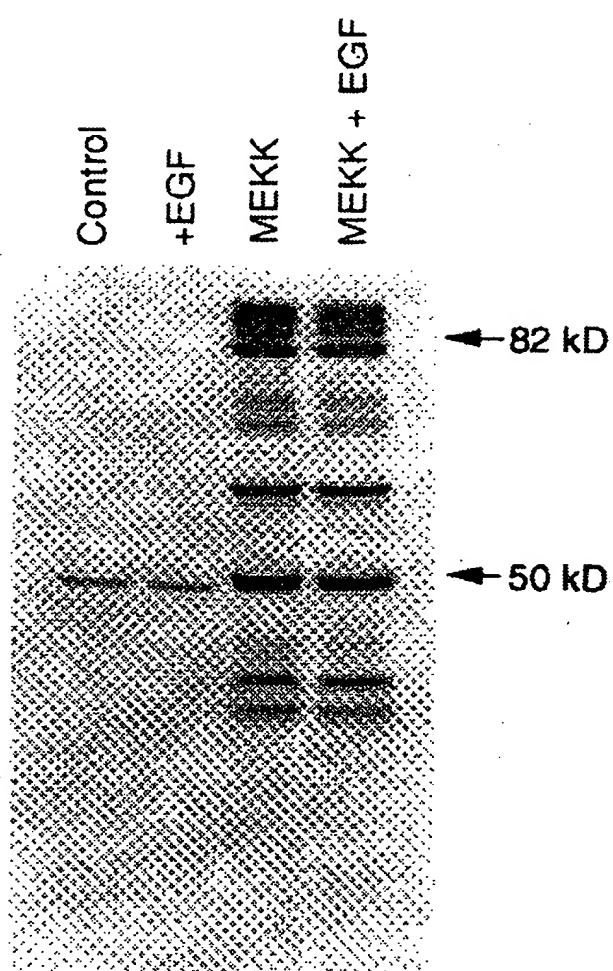


Fig. 6B

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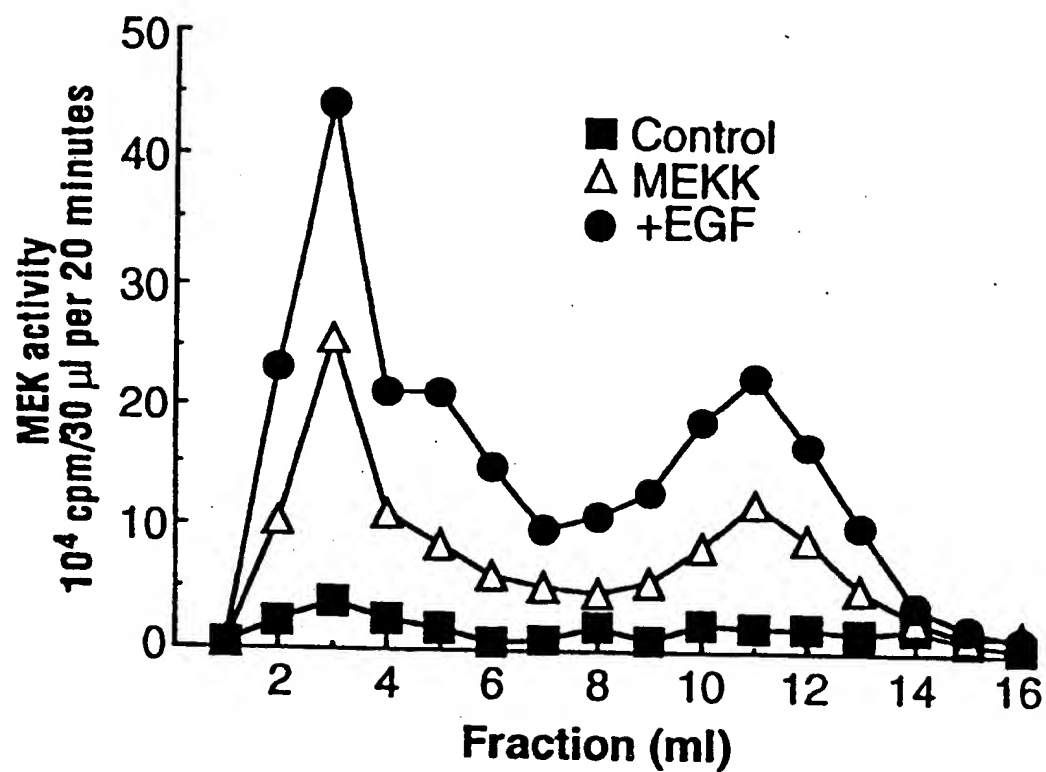


FIG. 7

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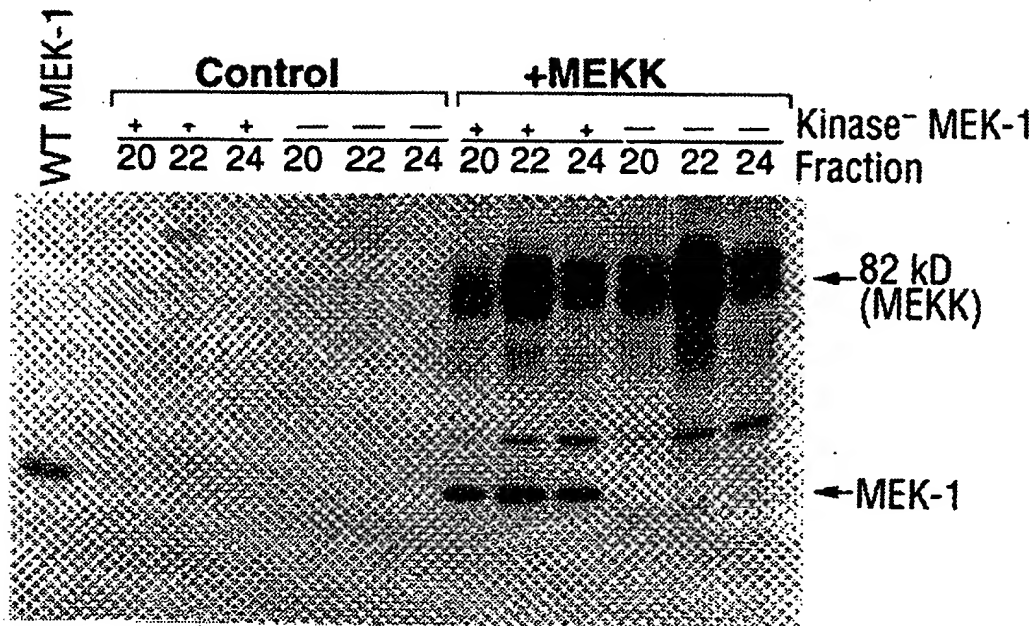


Fig. 8A

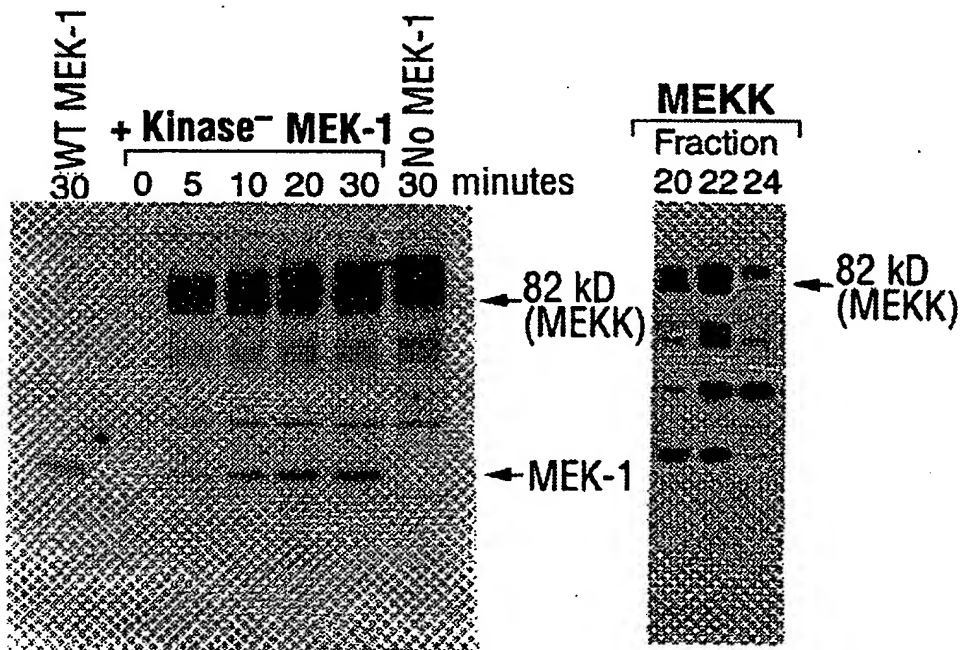


Fig. 8B

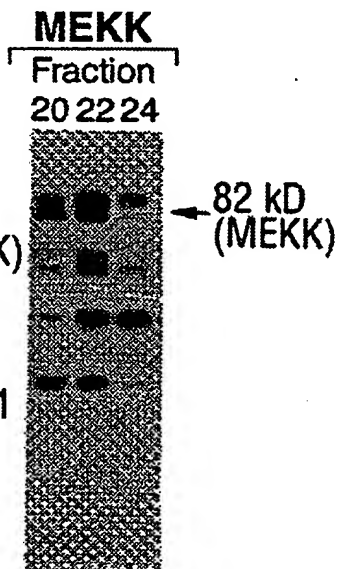


Fig. 8C

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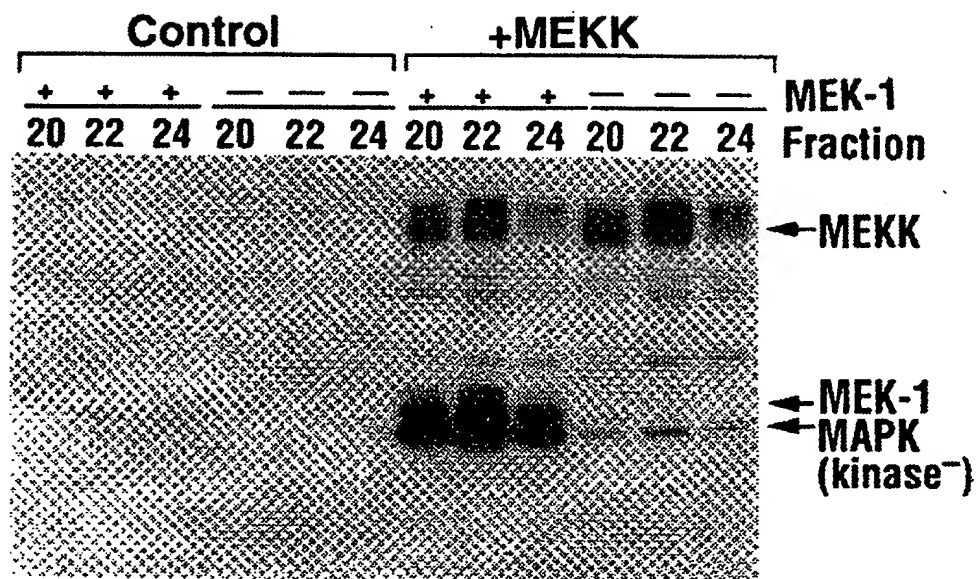


Fig. 9A

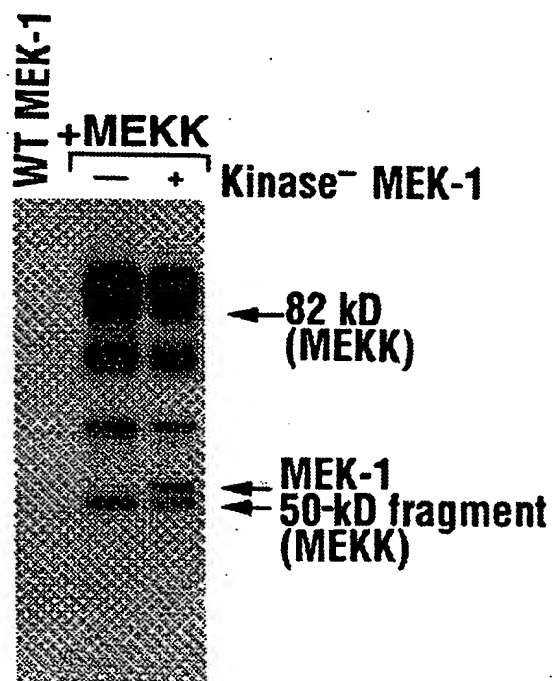


Fig. 9B

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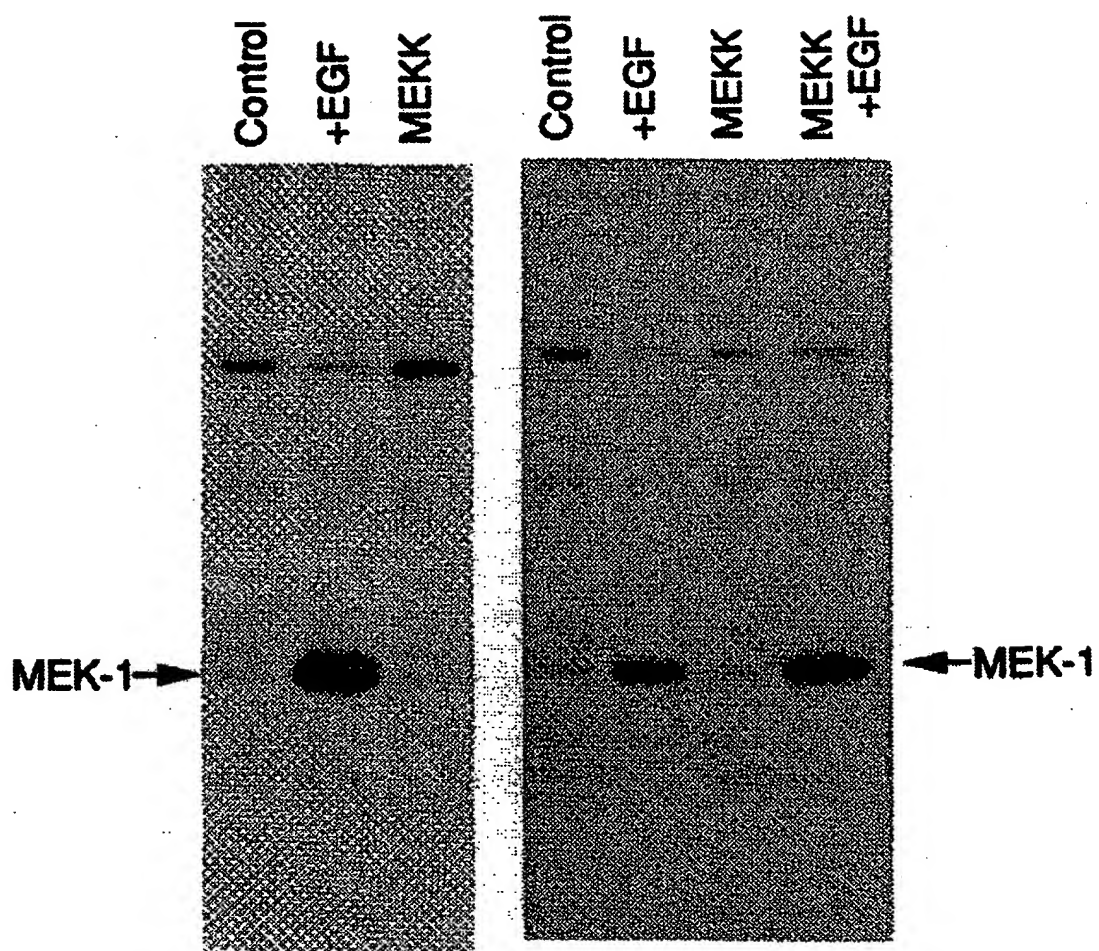


Fig. 10A

Fig. 10B

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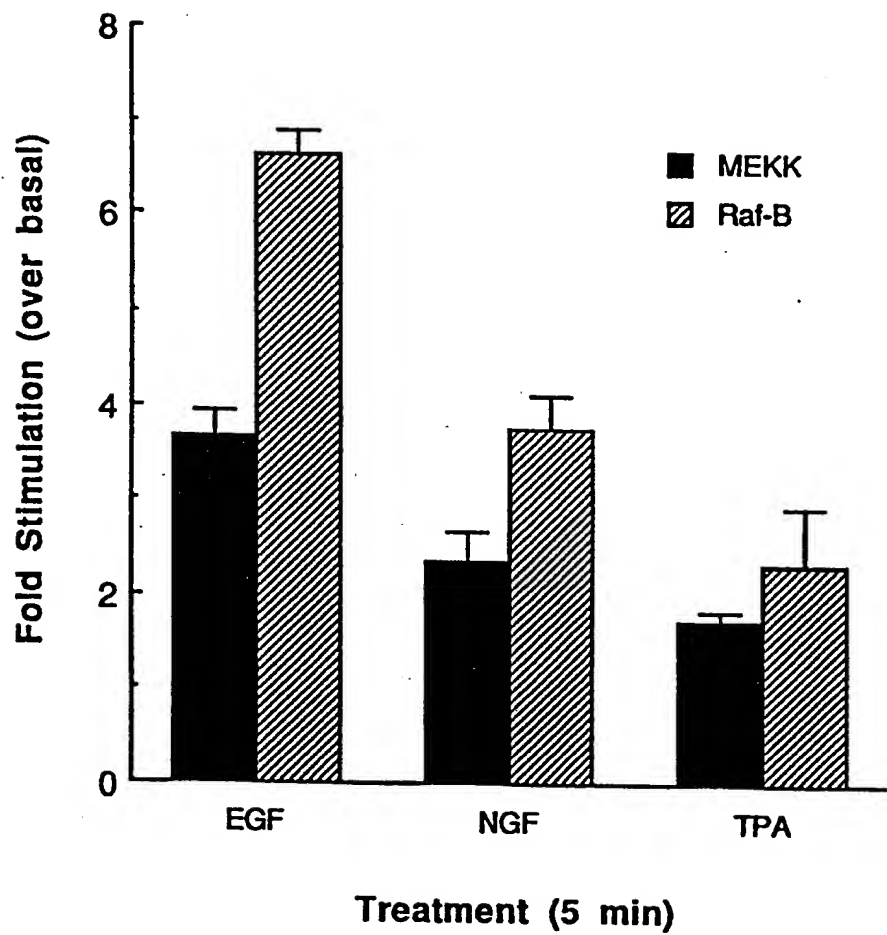


FIG. 11

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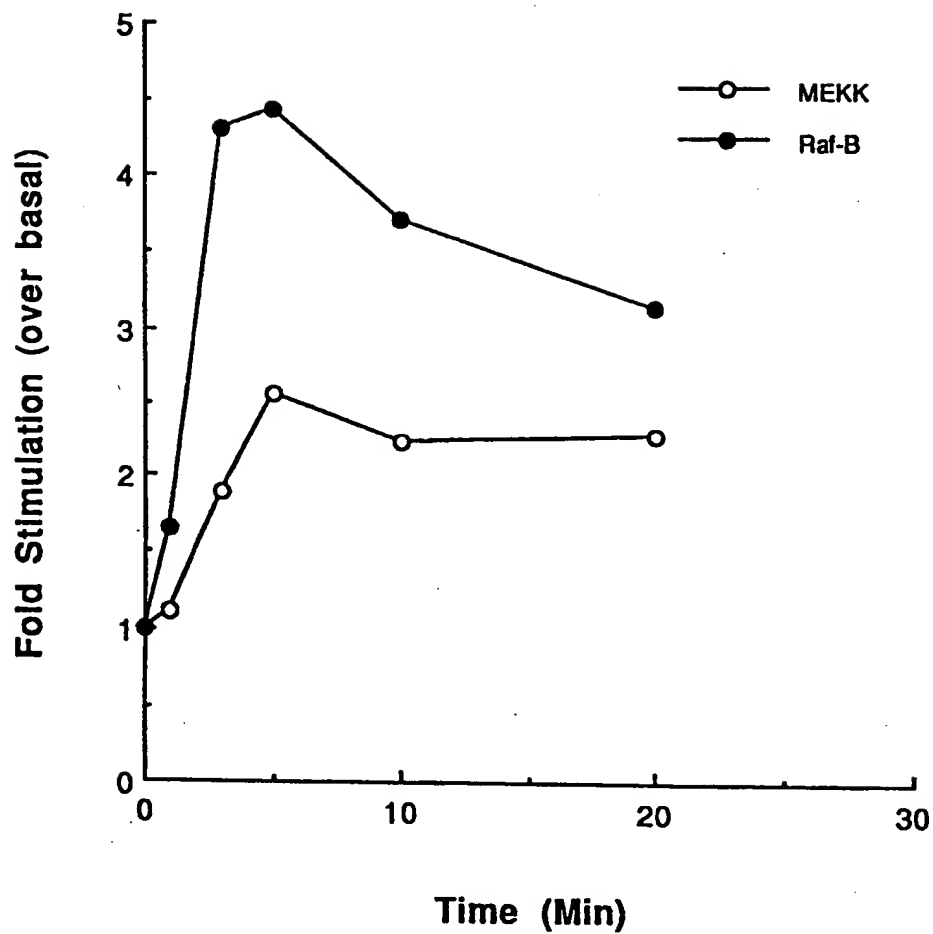


FIG. 12

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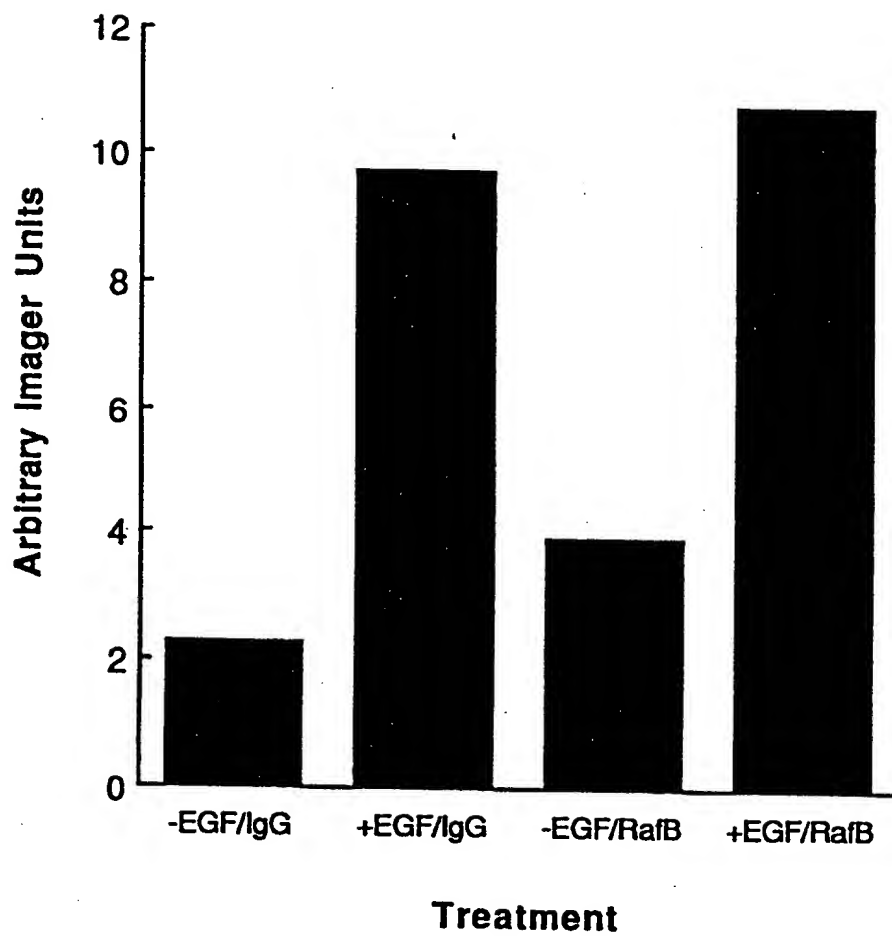


FIG. 13

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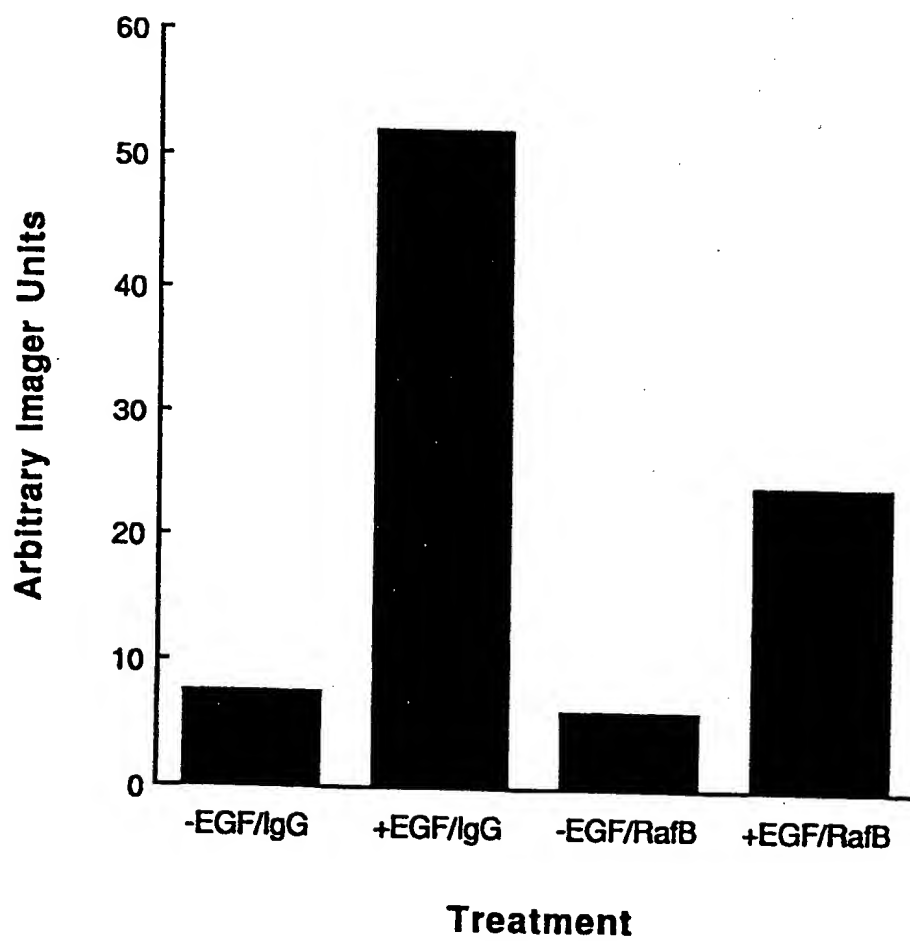


FIG. 14

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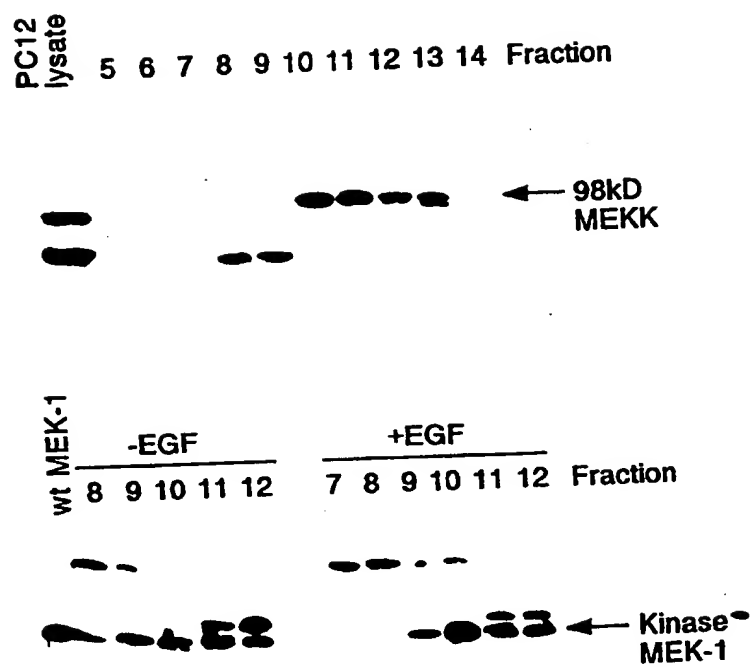


Fig. 15

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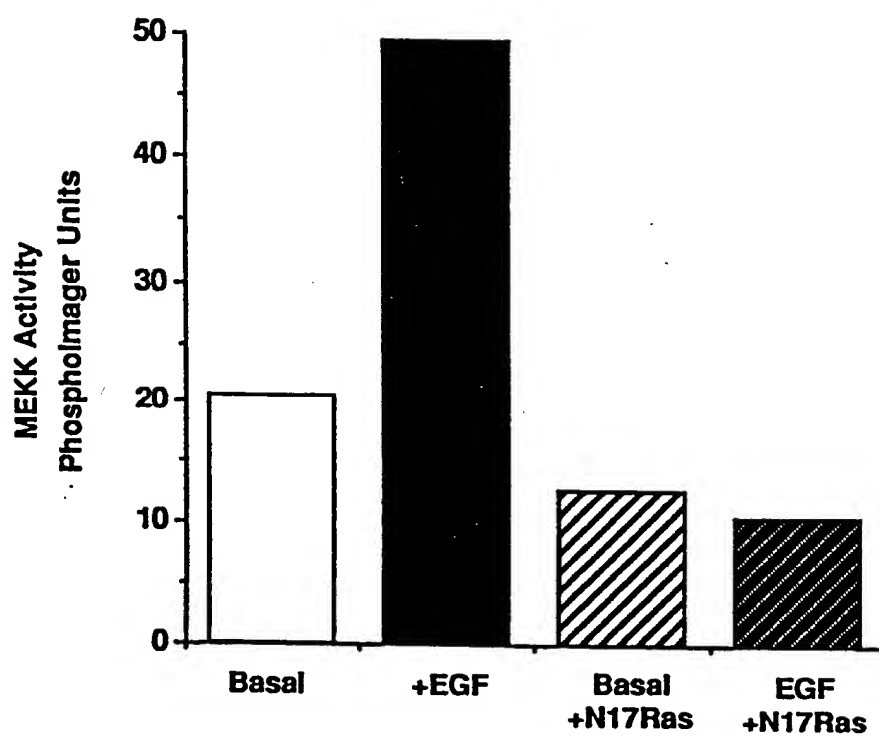


FIG. 16

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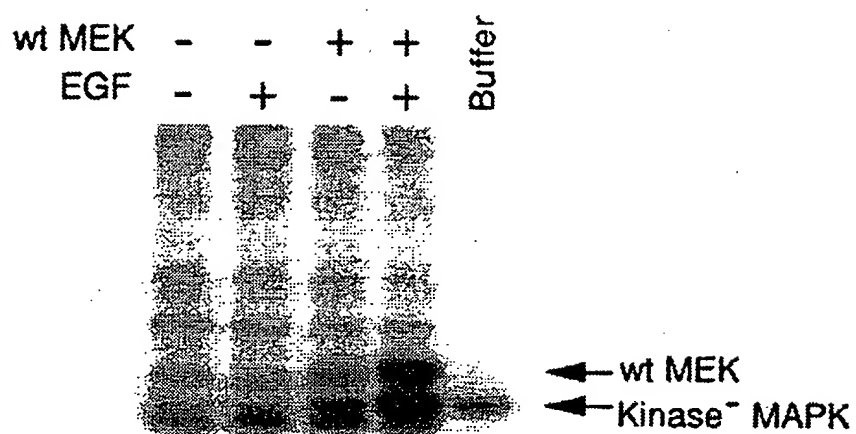


Fig. 17A

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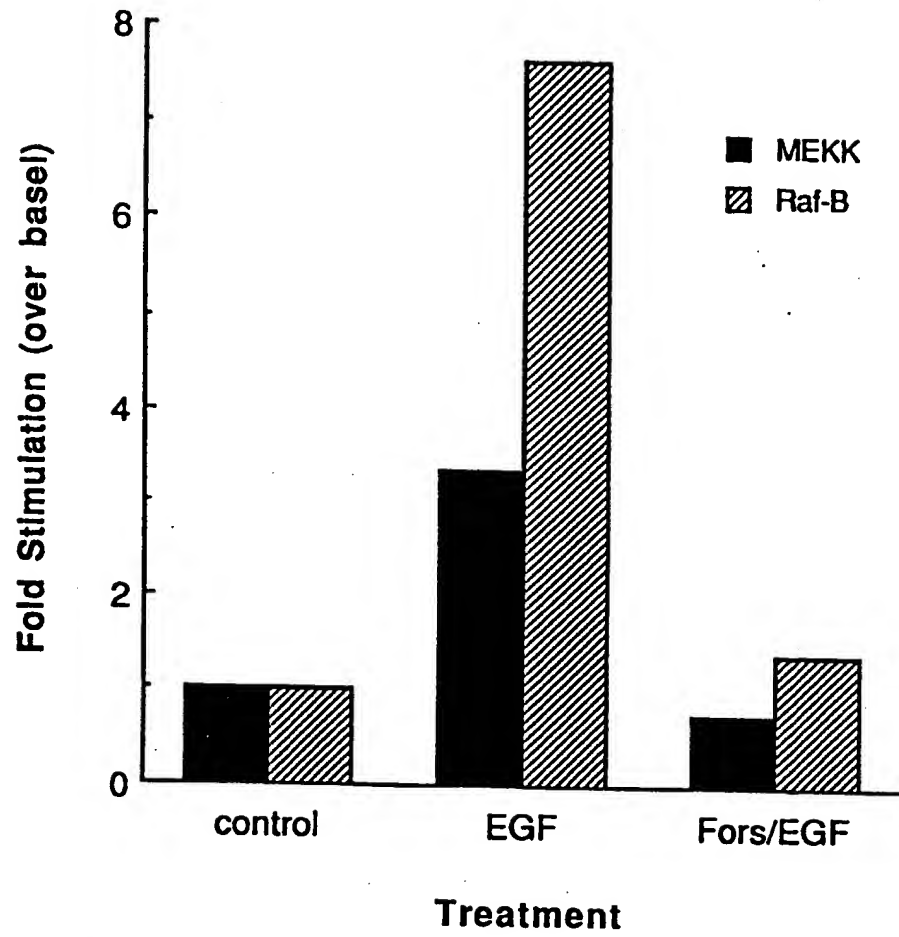


FIG. 18

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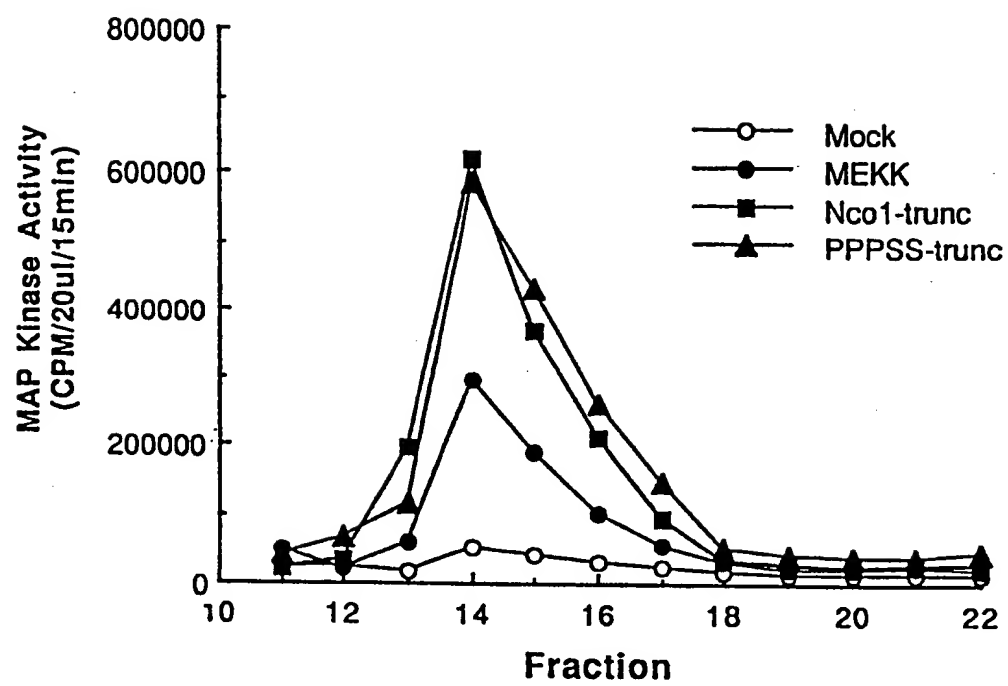


FIG. 19

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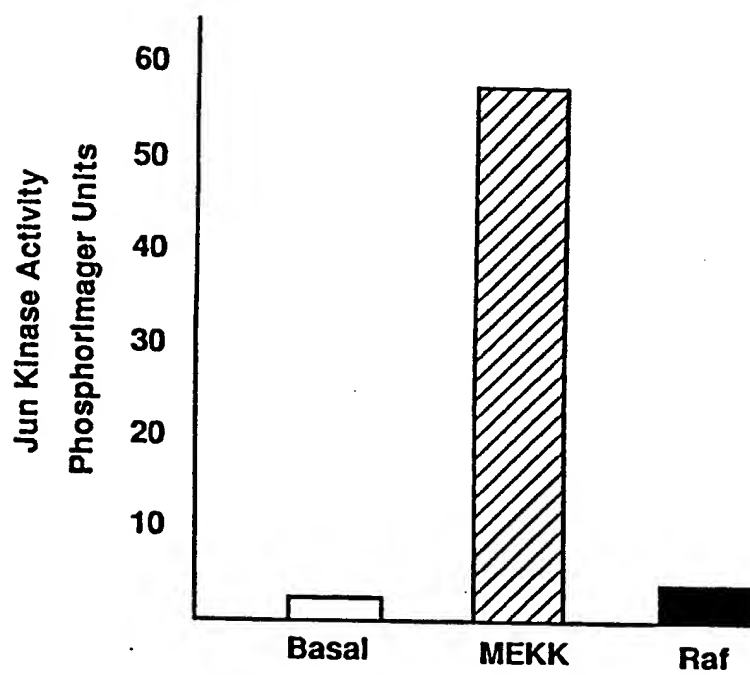


FIG. 20

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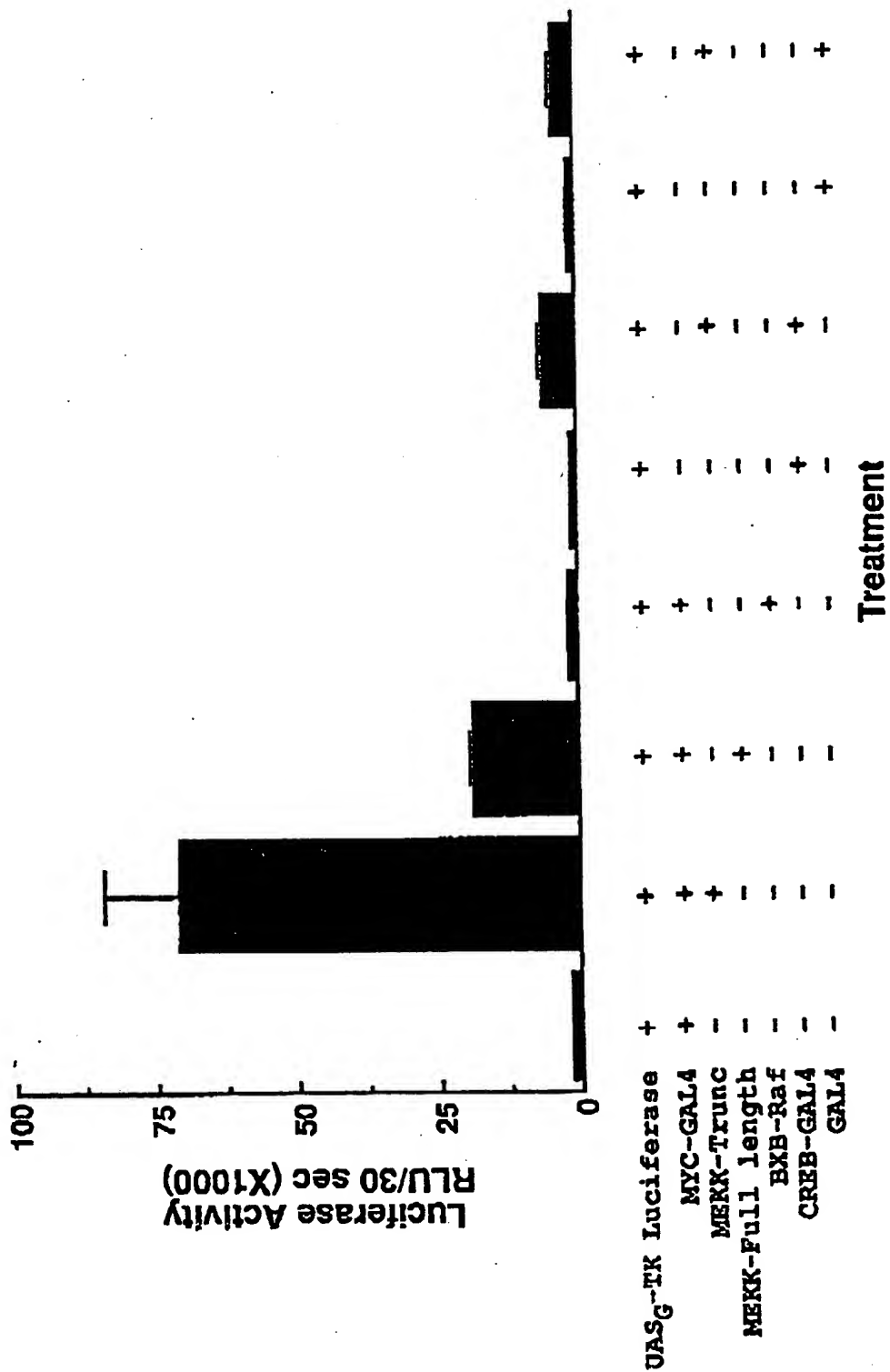
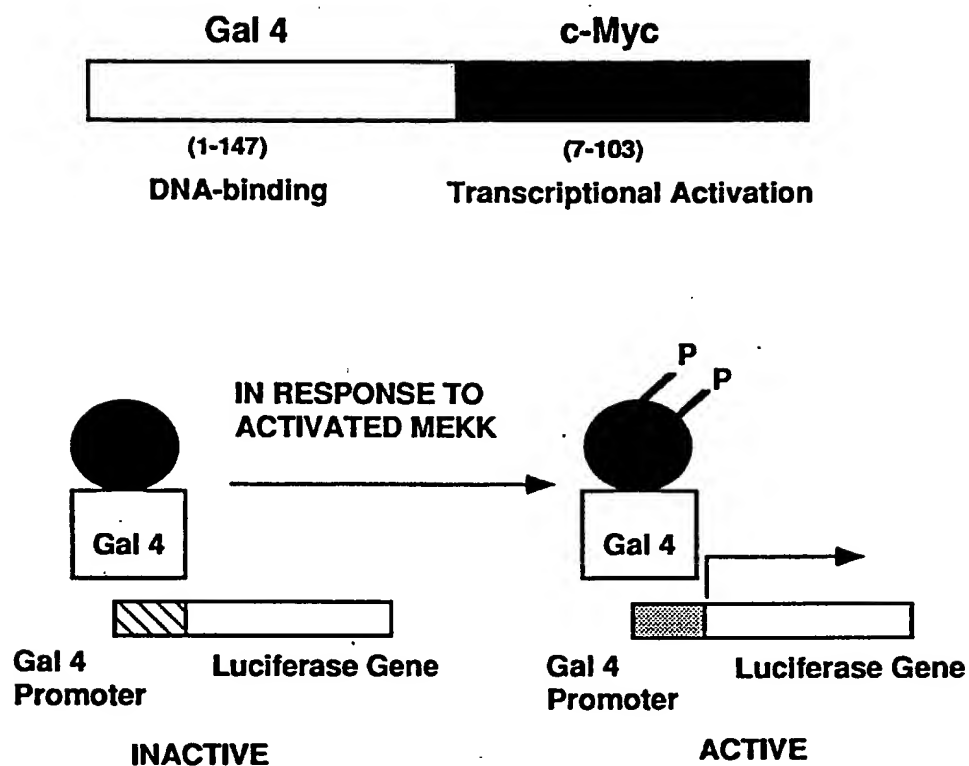


FIG. 21

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Myc-Gal 4 fusion protein:

**PHOSPHORYLATION OF C-MYC TRANSACTIVATION
DOMAIN IN RESPONSE TO MEKK EXPRESSION
ACTIVATES MYC-GAL 4 TRANSCRIPTIONAL ACTIVITY**

FIG. 22

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p42 → — — — ← p44

pCMV5 Control Vector

p42 → — — — ← p44
 — — — ← p38

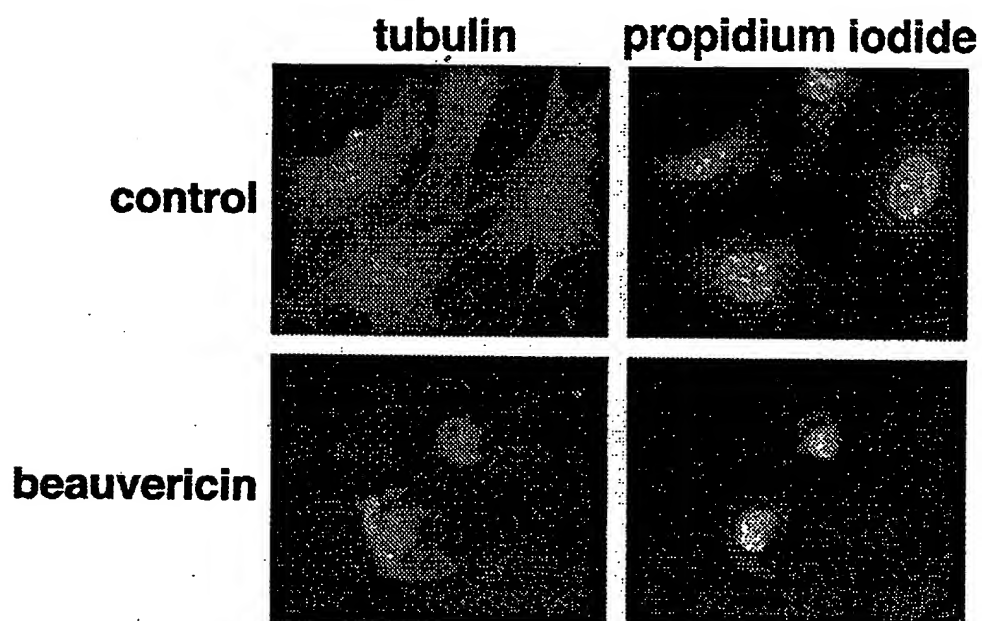
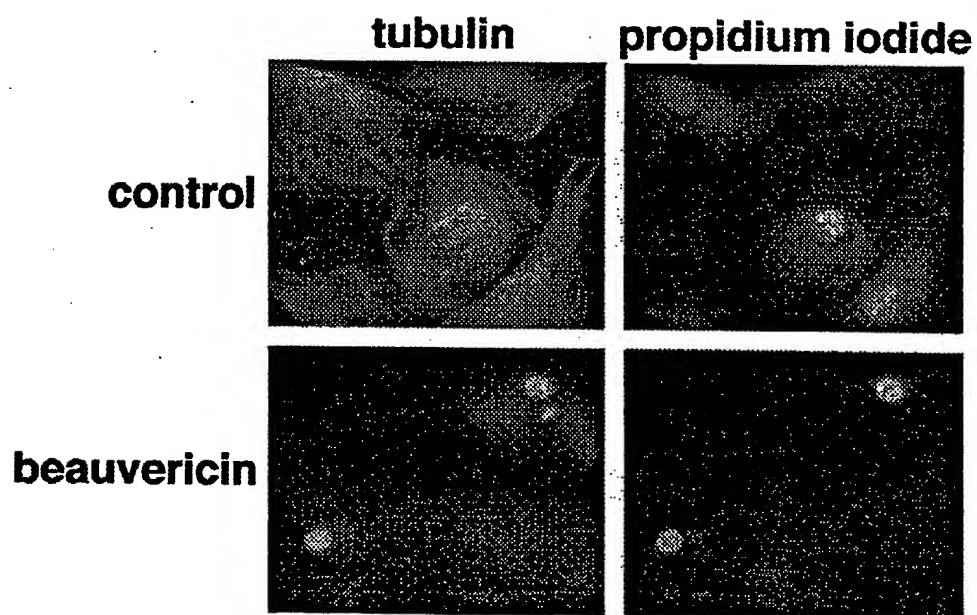
pCMV5-MEKK1

p42 → — — — ← p44
 — — — ← p38

pCMV5-MEKK3

Fig. 23

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Swiss 3T3**REF52****FIG. 24**

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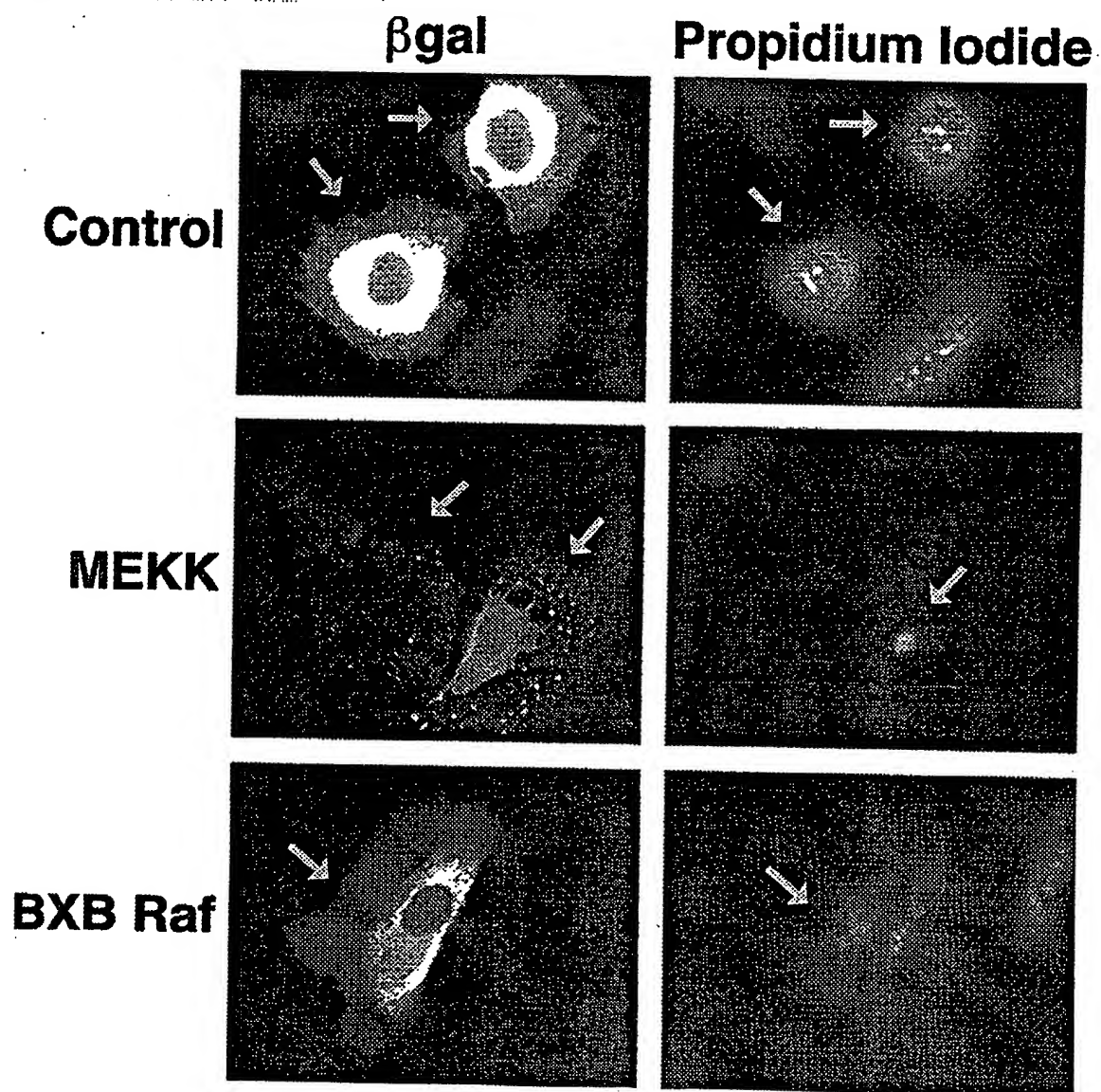


FIG. 25

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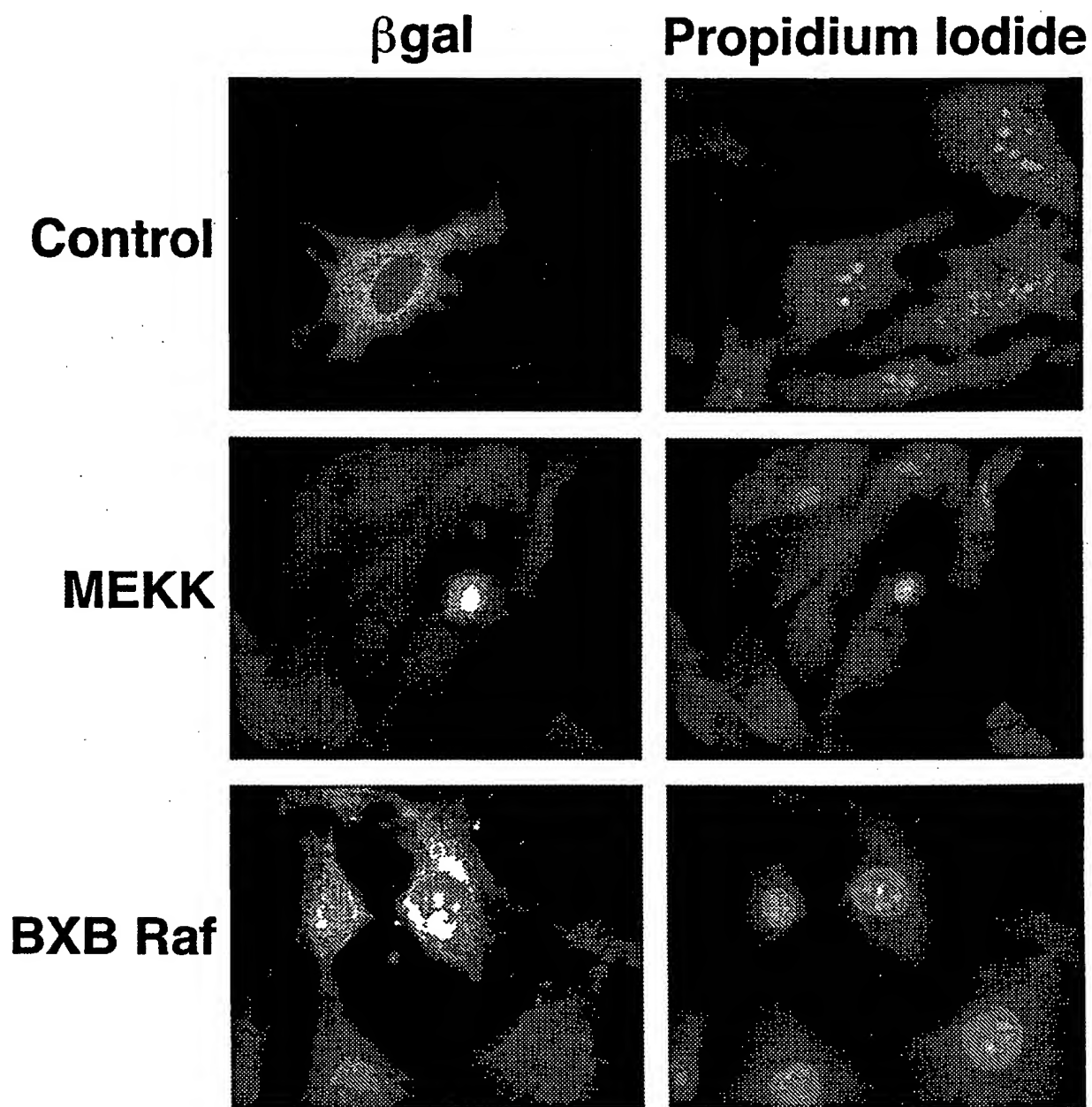


FIG. 26

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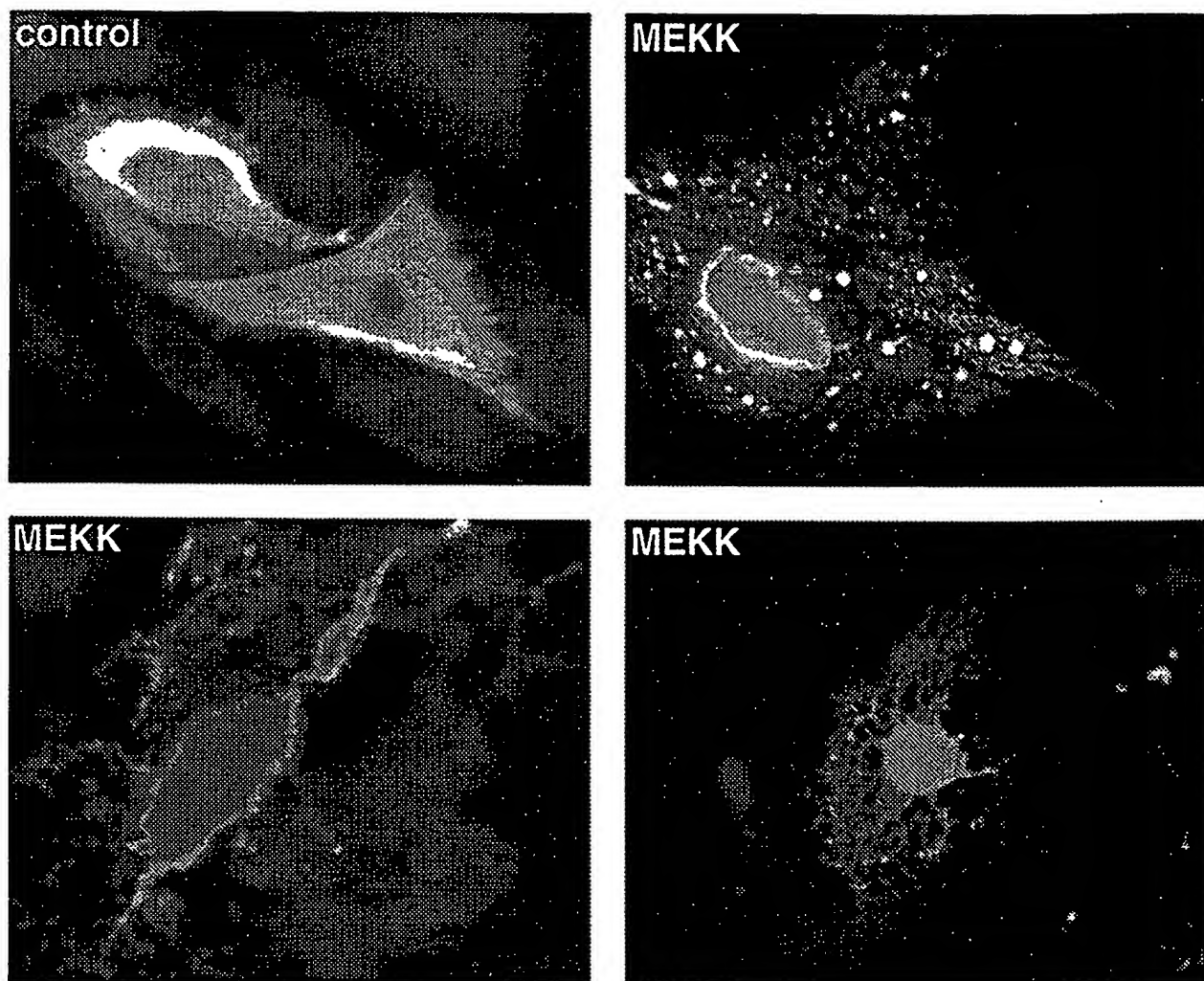


FIG. 27

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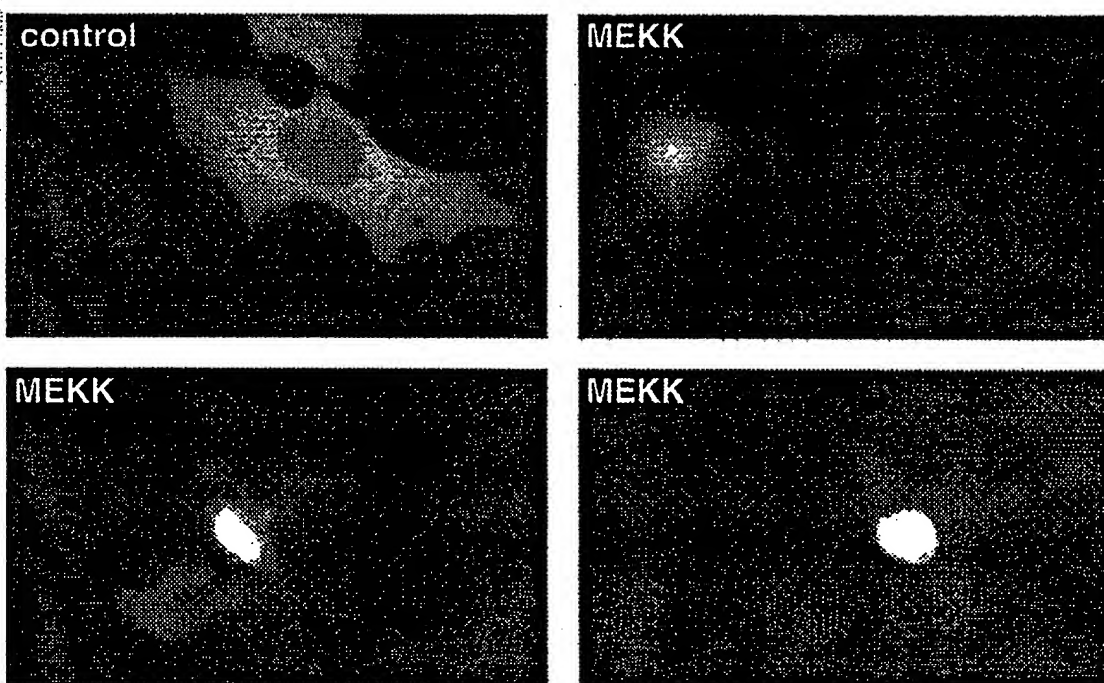


FIG. 28

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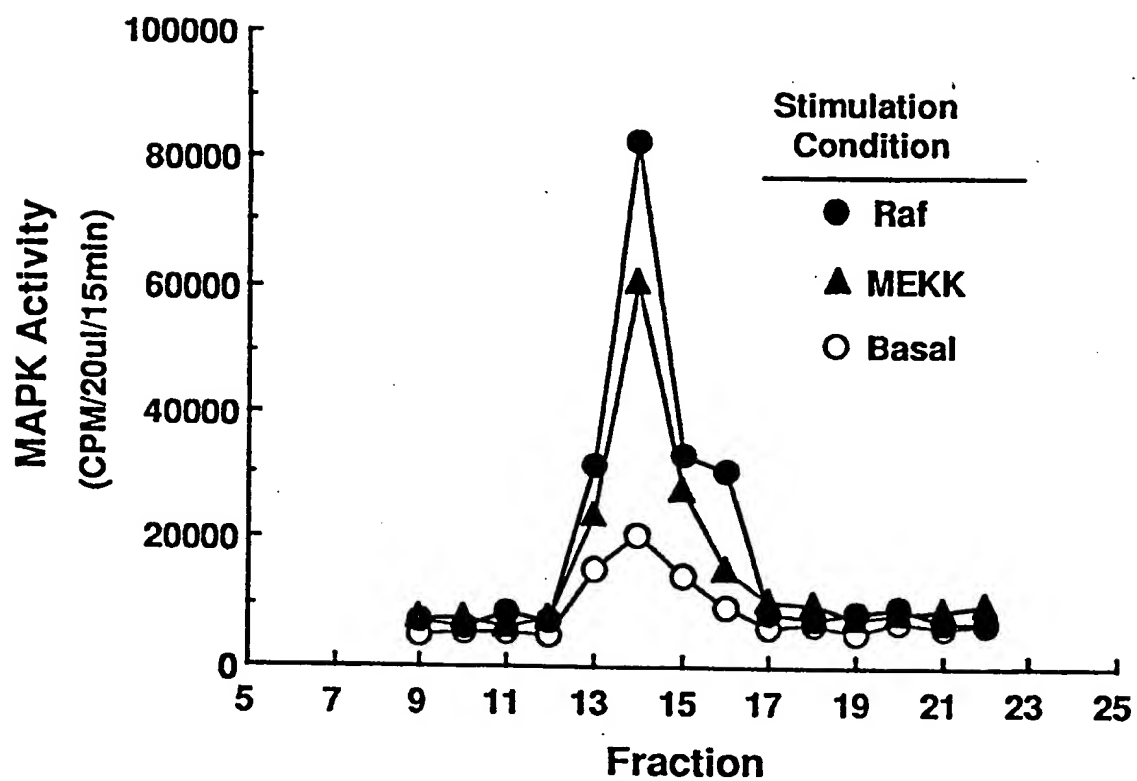


FIG. 29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/11690

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 14/00; C12N 15/00; C12Q 1/00; A61K 38/17

US CL : 435/4, 69.1, 240.1; 514/12; 530/350; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 69.1, 240.1; 514/12; 530/350; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, Medline, WPI

search terms: MEKK, MEK, Mapk kinase, raf protein, signal regulation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 258, issued 16 October 1992, Crews et al, "The Primary Structure of MEK, a Protein Kinase that Phosphorylates the <i>ERK</i> Gene Product", pages 478-480, see entire document.	1-10
Y	Molecular and Cellular Biology, Volume 5, Number 8, issued August 1985, Chaleff et al, "Molecular Cloning and Characterization of the <i>STE7</i> and <i>STE11</i> Genes of <i>Saccharomyces cerevisiae</i> ", pages 1878-1886, see pages 1880-1885.	1-10
Y	Science, Volume 257, issued 04 September 1992, Dent et al, "Activation of Mitogen-Activated Protein Kinase Kinase by v-Raf in NIH 3T3 Cells and in Vitro", pages 1404-1407, see entire document.	1-10

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be of particular relevance	* X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* &* document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means	
* P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

24 JANUARY 1995

Date of mailing of the international search report

08 FEB 1995

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/11690

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 260, issued 16 April 1993, Lange-Carter et al, "A Divergence in the MAP Kinase Regulatory Network Defined by MEK Kinase and Raf", pages 315-319, see entire document.	1-10
Y	Nature, Volume 358, issued 30 July 1992, Kyriakis et al, "Raf-1 Activates MAP Kinase-Kinase", pages 417-421, see page 419.	1-10
Y	Molecular and Cellular Biology, Volume 11, Number 7, issued July 1991, Wang et al, "byr2, a <i>Schizosaccharomyces pombe</i> Gene Encoding a Protein Kinase Capable of Partial Suppression of the <i>ras1</i> Mutant Phenotype", pages 3554-3563, see pages 3556-3561.	1-10